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(71) Applicant: RHONE-POULENC AGROCHIMIE [FR/FR]; 14-20, rue Pierre-Baizet, F-69263 Lyon (FR).

(72) Inventors: THOMAS, Terry, L.; 3004 Normand, College Station, TX 77845 (US). REDDY, Avutu, S.; 3902 E. 29th Street #G11, Bryan, TX 77802 (US). NUCCIO, Michael; P.O. Box 553, College Station, TX 77841 (US). NUNBERG, Andrew, N.; 2804 B. Sprucewood Street, Bryan, TX 77801 (US). FREYSSINET, Georges, L.; 21, rue de Nervleux, F-69450 Saint-Cyr-au-Mont-d'Or (FR).

(74) Agent: MITSCHERLICH & PARTNER; Sonnenstrasse 33, D-80331 München (DE).

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(54) Title: PRODUCTION OF GAMMA LINOLENIC ACID BY A Δ6-DESATURASE

(57) Abstract

Linoleic acid is converted into γ-linolenic acid by the enzyme Δ6-desaturase. The present invention is directed to isolated nucleic acids comprising the Δ6-desaturase gene. More particularly, the isolated nucleic acid comprises the promoter, coding region and termination regions of the Δ6-desaturase gene. The present invention provides recombinant constructions comprising the Δ6-desaturase coding region in functional combination with heterologous regulatory sequences. The nucleic acids and recombinant constructions of the instant invention are useful in the production of GLA in transgenic organisms.

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PRODUCTION OF GAMMA LINOLENIC ACID BY A Δ6-DESATURASE

Linoleic acid (18:2) (LA) is transformed into gamma linolenic acid (18:3) (GLA) by the enzyme Δ6-desaturase. When this enzyme, or the nucleic acid encoding it, is transferred into LA-producing cells, GLA is produced. The present invention provides nucleic acids comprising the Δ6-desaturase gene. More specifically, the nucleic acids comprise the promoters, coding regions and termination regions of the Δ6-desaturase genes. The present invention is further directed to recombinant constructions comprising a Δ6-desaturase coding region in functional combination with heterologous regulatory sequences. The nucleic acids and recombinant constructions of the instant invention are useful in the production of GLA in transgenic organisms.

Unsaturated fatty acids such as linoleic ($C_{18}\Delta^{9,12}$) and α-linolenic ($C_{18}\Delta^{9,12,15}$) acids are essential dietary constituents that cannot be synthesized by vertebrates since vertebrate cells can introduce double bonds at the Δ^9 position of fatty acids but cannot introduce additional double bonds between the Δ^9 double bond and the methyl-terminus of the fatty acid chain. Because they are precursors of other products, linoleic and α-linolenic acids are essential fatty acids, and are usually obtained from plant sources. Linoleic acid can be converted by mammals into γ-linolenic acid (GLA, $C_{18}\Delta^{6,9,12}$) which can in turn be converted to arachidonic acid (20:4), a critically

important fatty acid since it is an essential precursor of most prostaglandins.

The dietary provision of linoleic acid, by virtue of its resulting conversion to GLA and arachidonic acid, satisfies the dietary need for GLA and arachidonic acid. However, a relationship has been demonstrated between consumption of saturated fats and health risks such as hypercholesterolemia, atherosclerosis and other clinical disorders which correlate with susceptibility to coronary disease, while the consumption of unsaturated fats has been associated with decreased blood cholesterol concentration and reduced risk of atherosclerosis. The therapeutic benefits of dietary GLA may result from GLA being a precursor to arachidonic acid and thus subsequently contributing to prostaglandin synthesis. Accordingly, consumption of the more unsaturated GLA, rather than linoleic acid, has potential health benefits. However, GLA is not present in virtually any commercially grown crop plant.

Linoleic acid is converted into GLA by the enzyme Δ6-desaturase. Δ6-desaturase, an enzyme of more than 350 amino acids, has a membrane-bound domain and an active site for desaturation of fatty acids. When this enzyme is transferred into cells which endogenously produce linoleic acid but not GLA, GLA is produced. The present invention, by providing the gene encoding Δ6-desaturase, allows the production of transgenic organisms which contain functional Δ6-desaturase and which produce GLA. In addition to

allowing production of large amounts of GLA, the present invention provides new dietary sources of GLA.

The present invention is directed to isolated Δ6-desaturase genes. Specifically, the isolated genes comprises the Δ6-desaturase promoters, coding regions, and termination regions.

The present invention is further directed to expression vectors comprising the Δ6-desaturase promoter, coding region and termination region.

Yet another aspect of this invention is directed to expression vectors comprising a Δ6-desaturase coding region in functional combination with heterologous regulatory regions, i.e. elements not derived from the Δ6-desaturase gene.

Cells and organisms comprising the vectors of the present invention, and progeny of such organisms, are also provided by the present invention.

A further aspect of the present invention provides isolated bacterial Δ6-desaturase. An isolated plant Δ6-desaturase is also provided.

Yet another aspect of this invention provides a method for producing plants with increased gamma linolenic acid content.

A method for producing chilling tolerant plants is also provided by the present invention.

Fig. 1 depicts the hydropathy profiles of the deduced amino acid sequences of Synechocystis Δ6-desaturase (Panel A) and Δ12-desaturase (Panel B). Putative membrane spanning regions are indicated by solid bars. Hydrophobic index was calculated for a

window size of 19 amino acid residues [Kyte, et al. (1982) J. Molec. Biol. 157].

Fig. 2 provides gas liquid chromatography profiles of wild type (Panel A) and transgenic (Panel B) Anabaena.

Fig. 3 is a diagram of maps of cosmid cSy75, cSy13 and Csy7 with overlapping regions and subclones. The origins of subclones of Csy75, Csy75-3.5 and Csy7 are indicated by the dashed diagonal lines. Restriction sites that have been inactivated are in parentheses.

Fig. 4 provides gas liquid chromatography profiles of wild type (Panel A) and transgenic (Panel B) tobacco.

Fig. 5A depicts the DNA sequence of a Δ-6 desaturase cDNA isolated from borage.

Fig. 5B depicts the protein sequence of the open reading frame in the isolated borage Δ-6 desaturase cDNA. Three amino acid motifs characteristic of desaturases are indicated and are, in order, lipid box, metal box 1, and metal box 2.

Fig. 6 is a dendrogram showing similarity of the borage Δ6-desaturase to other membrane-bound desaturases. The amino acid sequence of the borage Δ6-desaturase was compared to other known desaturases using Gene Works (IntelliGenetics). Numerical values correlate to relative phylogenetic distances between subgroups compared.

Fig. 7 is a restriction map of 221.Δ6.NOS and 121.Δ6.NOS. In 221.Δ6.NOS, the remaining portion

of the plasmid is pBI221 and in 121.Δ6.NOS, the remaining portion of the plasmid is pBI121.

Fig. 8 provides gas liquid chromatography profiles of mock transfected (Panel A) and 221.Δ6.NOS transfected (Panel B) carrot cells. The positions of 18:2, 18:3 α, and 18:3 γ(GLA) are indicated.

Fig. 9 provides gas liquid chromatography profiles of an untransformed tobacco leaf (Panel A) and a tobacco leaf transformed with 121.Δ6.NOS. The positions of 18:2, 18:3 α, 18:3γ(GLA), and 18:4 are indicated.

Fig. 10 provides gas liquid chromotography profiles for untransformed tobacco seeds (Panel A) and seeds of tobacco transformed with 121.Δ6.NOS. The positions of 18:2, 18:3α and 18:3γ(GLA) are indicated.

The present invention provides isolated nucleic acids encoding Δ6-desaturase. To identify a nucleic acid encoding Δ6-desaturase, DNA is isolated from an organism which produces GLA. Said organism can be, for example, an animal cell, certain fungi (e.g. Mortierella), certain bacteria (e.g. Synechocystis) or certain plants (borage, Oenothera, currants). The isolation of genomic DNA can be accomplished by a variety of methods well-known to one of ordinary skill in the art, as exemplified by Sambrook et al. (1989) in Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY. The isolated DNA is fragmented by physical methods or enzymatic digestion and cloned into an appropriate vector, e.g. a bacteriophage or cosmid vector, by any of a variety of well-known methods which can be found

in references such as Sambrook et al. (1989). Expression vectors containing the DNA of the present invention are specifically contemplated herein. DNA encoding Δ6-desaturase can be identified by gain of function analysis. The vector containing fragmented DNA is transferred, for example by infection, transconjugation, transfection, into a host organism that produces linoleic acid but not GLA. As used herein, "transformation" refers generally to the incorporation of foreign DNA into a host cell. Methods for introducing recombinant DNA into a host organism are known to one of ordinary skill in the art and can be found, for example, in Sambrook et al. (1989). Production of GLA by these organisms (i.e., gain of function) is assayed, for example by gas chromatography or other methods known to the ordinarily skilled artisan. Organisms which are induced to produce GLA, i.e. have gained function by the introduction of the vector, are identified as expressing DNA encoding Δ6-desaturase, and said DNA is recovered from the organisms. The recovered DNA can again be fragmented, cloned with expression vectors, and functionally assessed by the above procedures to define with more particularity the DNA encoding Δ6-desaturase.

As an example of the present invention, random DNA is isolated from the cyanobacteria Synechocystis Pasteur Culture Collection (PCC) 6803, American Type Culture Collection (ATCC) 27184, cloned into a cosmid vector, and introduced by transconjugation into the GLA-deficient cyanobacterium

Anabaena strain PCC 7120, ATCC 27893. Production of GLA from Anabaena linoleic acid is monitored by gas chromatography and the corresponding DNA fragment is isolated.

The isolated DNA is sequenced by methods well-known to one of ordinary skill in the art as found, for example, in Sambrook et al. (1989).

In accordance with the present invention, DNA molecules comprising Δ6-desaturase genes have been isolated. More particularly, a 3.588 kilobase (kb) DNA comprising a Δ6-desaturase gene has been isolated from the cyanobacteria Synechocystis. The nucleotide sequence of the 3.588 kb DNA was determined and is shown in SEQ ID NO:1. Open reading frames defining potential coding regions are present from nucleotide 317 to 1507 and from nucleotide 2002 to 3081. To define the nucleotides responsible for encoding Δ6-desaturase, the 3.588 kb fragment that confers Δ6-desaturase activity is cleaved into two subfragments, each of which contains only one open reading frame. Fragment ORF1 contains nucleotides 1 through 1704, while fragment ORF2 contains nucleotides 1705 through 3588. Each fragment is subcloned in both forward and reverse orientations into a conjugal expression vector (AM542, Wolk et al. [1984] Proc. Natl. Acad. Sci. USA 81, 1561) that contains a cyanobacterial carboxylase promoter. The resulting constructs (i.e. ORF1(F), ORF1(R), ORF2(F) and ORF2(R)] are conjugated to wild-type Anabaena PCC 7120 by standard methods (see, for example, Wolk et al. (1984) Proc. Natl. Acad. Sci. USA 81, 1561). Conjugated cells of Anabaena are

identified as Neo^R green colonies on a brown background of dying non-conjugated cells after two weeks of growth on selective media (standard mineral media BG11N + containing 30μg/ml of neomycin according to Rippka et al., (1979) J. Gen Microbiol. 111, 1). The green colonies are selected and grown in selective liquid media (BG11N + with 15μg/ml neomycin). Lipids are extracted by standard methods (e.g. Dahmer et al., (1989) Journal of American Oil Chemical Society 66, 543) from the resulting transconjugants containing the forward and reverse oriented ORF1 and ORF2 constructs. For comparison, lipids are also extracted from wild-type cultures of *Anabaena* and *Synechocystis*. The fatty acid methyl esters are analyzed by gas liquid chromatography (GLC), for example with a Tracor-560 gas liquid chromatograph equipped with a hydrogen flame ionization detector and a capillary column. The results of GLC analysis are shown in Table 1.

Table 1: Occurrence of C18 fatty acids in wild-type and transgenic cyanobacteria

SOURCE	18:0	18:1	18:2	γ18:3	α18:3	18:4
Anabaena (wild type)	+	+	+	-	+	-
Anabaena + ORF1(F)	+	+	+	-	+	-
Anabaena + ORF1(R)	+	+	+	-	+	-
Anabaena + ORF2(F)	+	+	+	+	+	+
Anabaena + ORF2(R)	+	+	+	-	+	-
Synechocystis (wild type)	+	+	+	+	-	-

As assessed by GLC analysis, GLA deficient <u>Anabaena</u> gain the function of GLA production when the construct containing ORF2 in forward orientation is introduced by transconjugation. Transconjugants containing constructs with ORF2 in reverse orientation to the carboxylase promoter, or ORF1 in either orientation, show no GLA production. This analysis demonstrates that the single open reading frame (ORF2) within the 1884 bp fragment encodes Δ6-desaturase. The 1884 bp fragment is shown as SEQ ID NO:3. This is substantiated by the overall similarity of the hydropathy profiles between Δ6-desaturase and Δ12-desaturase [Wada <u>et al</u>. (1990) <u>Nature</u> 347] as shown in Fig. 1 as (A) and (B), respectively.

Also in accordance with the present invention, a cDNA comprising a Δ6-desaturase gene from borage (<u>Borago officinalis</u>) has been isolated. The nucleotide sequence of the 1.685 kilobase (kb) cDNA

was determined and is shown in Fig. 5A (SEQ ID NO: 4). The ATG start codon and stop codon are underlined. The amino acid sequence corresponding to the open reading frame in the borage delta 6-desaturase is shown in Fig. 5B (SEQ ID NO: 5).

Isolated nucleic acids encoding Δ6-desaturase can be identified from other GLA-producing organisms by the gain of function analysis described above, or by nucleic acid hybridization techniques using the isolated nucleic acid which encodes Synechocystis or borage Δ6-desaturase as a hybridization probe. Both genomic and cDNA cloning methods are known to the skilled artisan and are contemplated by the present invention. The hybridization probe can comprise the entire DNA sequence disclosed as SEQ. ID NO:1 or SEQ. ID NO:4, or a restriction fragment or other DNA fragment thereof, including an oligonucleotide probe. Methods for cloning homologous genes by cross-hybridization are known to the ordinarily skilled artisan and can be found, for example, in Sambrook (1989) and Beltz et al. (1983) Methods in Enzymology 100, 266.

In another method of identifying a delta 6-desaturase gene from an organism producing GLA, a cDNA library is made from poly-A^{+} RNA isolated from polysomal RNA. In order to eliminate hyper-abundant expressed genes from the cDNA population, cDNAs or fragments thereof corresponding to hyper-abundant cDNAs genes are used as hybridization probes to the cDNA library. Non hybridizing plaques are excised and the resulting bacterial colonies are used to inoculate

liquid cultures and sequenced. For example, as a means of eliminating other seed storage protein cDNAs from a cDNA library made from borage polysomal RNA, cDNAs corresponding to abundantly expressed seed storage proteins are first hybridized to the cDNA library. The "subtracted" DNA library is then used to generate expressed sequence tags (ETSs) and such tags are used to scan a data base such as GenBank to identify potential desaturates.

Transgenic organisms which gain the function of GLA production by introduction of DNA encoding Δ-desaturase also gain the function of octadecatetraeonic acid ($18{:}4^{\Delta 6,9,12,15}$) production. Octadecatetraeonic acid is present normally in fish oils and in some plant species of the Boraginaceae family (Craig et al. [1964] J. Amer. Oil Chem. Soc. 41, 209-211; Gross et al. [1976] Can. J. Plant Sci. 56, 659-664). In the transgenic organisms of the present invention, octadecatetraenoic acid results from further desaturation of α-linolenic acid by Δ6-desaturase or desaturation of GLA by Δ15-desaturase.

The 359 amino acids encoded by ORF2, i.e. the open reading frame encoding Synechocystis Δ6-desaturase, are shown as SEQ. ID NO:2. The open reading frame encoding the borage Δ6-desaturase is shown in SEQ ID NO: 5. The present invention further contemplates other nucleotide sequences which encode the amino acids of SEQ ID NO:2 and SEQ ID NO: 5. It is within the ken of the ordinarily skilled artisan to identify such sequences which result, for example, from the degeneracy of the genetic code. Furthermore,

one of ordinary skill in the art can determine, by the gain of function analysis described hereinabove, smaller subfragments of the fragments containing the open reading frames which encode Δ6-desaturases.

The present invention contemplates any such polypeptide fragment of Δ6-desaturase and the nucleic acids therefor which retain activity for converting LA to GLA.

In another aspect of the present invention, a vector containing a nucleic acid of the present invention or a smaller fragment containing the promoter, coding sequence and termination region of a Δ6-desaturase gene is transferred into an organism, for example, cyanobacteria, in which the Δ6-desaturase promoter and termination regions are functional. Accordingly, organisms producing recombinant Δ6-desaturase are provided by this invention. Yet another aspect of this invention provides isolated Δ6-desaturase, which can be purified from the recombinant organisms by standard methods of protein purification. (For example, see Ausubel et al. [1987] Current Protocols in Molecular Biology, Green Publishing Associates, New York).

Vectors containing DNA encoding Δ6-desaturase are also provided by the present invention. It will be apparent to one of ordinary skill in the art that appropriate vectors can be constructed to direct the expression of the Δ6-desaturase coding sequence in a variety of organisms. Replicable expression vectors are particularly preferred. Replicable expression vectors as described herein are

DNA or RNA molecules engineered for controlled expression of a desired gene, i.e. the Δ6-desaturase gene. Preferably the vectors are plasmids, bacteriophages, cosmids or viruses. Shuttle vectors, e.g. as described by Wolk et al. (1984) Proc. Natl. Acad. Sci. USA, 1561-1565 and Bustos et al. (1991) J. Bacteriol. 174, 7525-7533, are also contemplated in accordance with the present invention. Sambrook et al. (1989), Goeddel, ed. (1990) Methods in Enzymology 185 Academic Press, and Perbal (1988) A Practical Guide to Molecular Cloning, John Wiley and Sons, Inc., provide detailed reviews of vectors into which a nucleic acid encoding the present Δ6-desaturase can be inserted and expressed. Such vectors also contain nucleic acid sequences which can effect expression of nucleic acids encoding Δ6-desaturase. Sequence elements capable of effecting expression of a gene product include promoters, enhancer elements, upstream activating sequences, transcription termination signals and polyadenylation sites. Both constitutive and tissue specific promoters are contemplated. For transformation of plant cells, the cauliflower mosaic virus (CaMV) 35S promoter and promoters which are regulated during plant seed maturation are of particular interest. All such promoter and transcriptional regulatory elements, singly or in combination, are contemplated for use in the present replicable expression vectors and are known to one of ordinary skill in the art. The CaMV 355 promoter is described, for example, by Restrepo et al. (1990)

Plant Cell 2, 987. Genetically engineered and mutated regulatory sequences are also contemplated.

The ordinarily skilled artisan can determine vectors and regulatory elements suitable for expression in a particular host cell. For example, a vector comprising the promoter from the gene encoding the carboxylase of Anabaena operably linked to the coding region of Δ6-desaturase and further operably linked to a termination signal from Synechocystis is appropriate for expression of Δ6-desaturase in cyanobacteria. "Operably linked" in this context means that the promoter and terminator sequences effectively function to regulate transcription. As a further example, a vector appropriate for expression of Δ6-desaturase in transgenic plants can comprise a seed-specific promoter sequence derived from helianthinin, napin, or glycinin operably linked to the Δ6-desaturase coding region and further operably linked to a seed termination signal or the nopaline synthase termination signal. As a still further example, a vector for use in expression of Δ 6-desaturase in plants can comprise a constitutive promoter or a tissue specific promoter operably linked to the Δ 6-desaturase coding region and further operably linked to a constitutive or tissue specific terminator or the nopaline synthase termination signal.

In particular, the helianthinin regulatory elements disclosed in applicant's copending U.S. Application Serial No. 682,354, filed April 8, 1991 and incorporated herein by reference, are contemplated

as promoter elements to direct the expression of the Δ6-desaturase of the present invention.

Modifications of the nucleotide sequences or regulatory elements disclosed herein which maintain the functions contemplated herein are within the scope of this invention. Such modifications include insertions, substitutions and deletions, and specifically substitutions which reflect the degeneracy of the genetic code.

Standard techniques for the construction of such hybrid vectors are well-known to those of ordinary skill in the art and can be found in references such as Sambrook _et al_. (1989), or any of the myriad of laboratory manuals on recombinant DNA technology that are widely available. A variety of strategies are available for ligating fragments of DNA, the choice of which depends on the nature of the termini of the DNA fragments. It is further contemplated in accordance with the present invention to include in the hybrid vectors other nucleotide sequence elements which facilitate cloning, expression or processing, for example sequences encoding signal peptides, a sequence encoding KDEL, which is required for retention of proteins in the endoplasmic reticulum or sequences encoding transit peptides which direct Δ6-desaturase to the chloroplast. Such sequences are known to one of ordinary skill in the art. An optimized transit peptide is described, for example, by Van den Broeck _et al_. (1985) _Nature_ _313_, 358. Prokaryotic and eukaryotic signal sequences are

disclosed, for example, by Michaelis et al. (1982) Ann. Rev. Microbiol. 36, 425.

A further aspect of the instant invention provides organisms other than cyanobacteria or plants which contain the DNA encoding the Δ6-desaturase of the present invention. The transgenic organisms contemplated in accordance with the present invention include bacteria, cyanobacteria, fungi, and plants and animals. The isolated DNA of the present invention can be introduced into the host by methods known in the art, for example infection, transfection, transformation or transconjugation. Techniques for transferring the DNA of the present invention into such organisms are widely known and provided in references such as Sambrook et al. (1989).

A variety of plant transformation methods are known. The Δ6-desaturase gene can be introduced into plants by a leaf disk transformation-regeneration procedure as described by Horsch et al. (1985) Science 227, 1229. Other methods of transformation, such as protoplast culture (Horsch et al. (1984) Science 223, 496; DeBlock et al. (1984) EMBO J. 2, 2143; Barton et al. (1983) Cell 32, 1033) can also be used and are within the scope of this invention. In a preferred embodiment plants are transformed with Agrobacterium-derived vectors. However, other methods are available to insert the Δ6-desaturase genes of the present invention into plant cells. Such alternative methods include biolistic approaches (Klein et al. (1987) Nature 327, 70), electroporation, chemically-induced DNA uptake, and use of viruses or pollen as vectors.

When necessary for the transformation method, the Δ6-desaturase genes of the present invention can be inserted into a plant transformation vector, e.g. the binary vector described by Bevan (1984) Nucleic Acids Res. 12, 8111. Plant transformation vectors can be derived by modifying the natural gene transfer system of *Agrobacterium tumefaciens*. The natural system comprises large Ti (tumor-inducing)-plasmids containing a large segment, known as T-DNA, which is transferred to transformed plants. Another segment of the Ti plasmid, the *vir* region, is responsible for T-DNA transfer. The T-DNA region is bordered by terminal repeats. In the modified binary vectors the tumor-inducing genes have been deleted and the functions of the *vir* region are utilized to transfer foreign DNA bordered by the T-DNA border sequences. The T-region also contains a selectable marker for antibiotic resistance, and a multiple cloning site for inserting sequences for transfer. Such engineered strains are known as "disarmed" *A. tumefaciens* strains, and allow the efficient transformation of sequences bordered by the T-region into the nuclear genomes of plants.

Surface-sterilized leaf disks are inoculated with the "disarmed" foreign DNA-containing *A. tumefaciens*, cultured for two days, and then transferred to antibiotic-containing medium. Transformed shoots are selected after rooting in medium containing the appropriate antibiotic, transferred to soil and regenerated.

Another aspect of the present invention provides transgenic plants or progeny of these plants containing the isolated DNA of the invention. Both monocotyledenous and dicotyledenous plants are contemplated. Plant cells are transformed with the isolated DNA encoding Δ6-desaturase by any of the plant transformation methods described above. The transformed plant cell, usually in a callus culture or leaf disk, is regenerated into a complete transgenic plant by methods well-known to one of ordinary skill in the art (e.g. Horsch _et al_. (1985) _Science_ _227_, 1129). In a preferred embodiment, the transgenic plant is sunflower, oil seed rape, maize, tobacco, peanut or soybean. Since progeny of transformed plants inherit the DNA encoding Δ6-desaturase, seeds or cuttings from transformed plants are used to maintain the transgenic plant line.

The present invention further provides a method for providing transgenic plants with an increased content of GLA. This method includes introducing DNA encoding Δ6-desaturase into plant cells which lack or have low levels of GLA but contain LA, and regenerating plants with increased GLA content from the transgenic cells. In particular, commercially grown crop plants are contemplated as the transgenic organism, including, but not limited to, sunflower, soybean, oil seed rape, maize, peanut and tobacco.

The present invention further provides a method for providing transgenic organisms which contain GLA. This method comprises introducing DNA

encoding Δ6-desaturase into an organism which lacks or has low levels of GLA, but contains LA. In another embodiment, the method comprises introducing one or more expression vectors which comprise DNA encoding Δ12-desaturase and Δ6-desaturase into organisms which are deficient in both GLA and LA. Accordingly, organisms deficient in both LA and GLA are induced to produce LA by the expression of Δ12-desaturase, and GLA is then generated due to the expression of Δ6-desaturase. Expression vectors comprising DNA encoding Δ12-desaturase, or Δ12-desaturase and Δ6-desaturase, can be constructed by methods of recombinant technology known to one of ordinary skill in the art (Sambrook et al., 1989) and the published sequence of Δ12-desaturase (Wada et al [1990] Nature (London) 347, 200-203. In addition, it has been discovered in accordance with the present invention that nucleotides 2002-3081 of SEQ. ID NO:1 encode cyanobacterial Δ12-desaturase. Accordingly, this sequence can be used to construct the subject expression vectors. In particular, commercially grown crop plants are contemplated as the transgenic organism, including, but not limited to, sunflower, soybean, oil seed rape, maize, peanut and tobacco.

The present invention is further directed to a method of inducing chilling tolerance in plants. Chilling sensitivity may be due to phase transition of lipids in cell membranes. Phase transition temperature depends upon the degree of unsaturation of fatty acids in membrane lipids, and thus increasing the degree of unsaturation, for example by introducing

Δ6-desaturase to convert LA to GLA, can induce or improve chilling resistance. Accordingly, the present method comprises introducing DNA encoding Δ6-desaturase into a plant cell, and regenerating a plant with improved chilling resistance from said transformed plant cell. In a preferred embodiment, the plant is a sunflower, soybean, oil seed rape, maize, peanut or tobacco plant.

The following examples further illustrate the present invention.

EXAMPLE 1

Strains and Culture Conditions

Synechocystis (PCC 6803, ATCC 27184), *Anabaena* (PCC 7120, ATCC 27893) and *Synechococcus* (PCC 7942, ATCC 33912) were grown photoautotrophically at 30°C in BG11N+ medium (Rippka *et al*. [1979] *J. Gen. Microbiol.* 111, 1-61) under illumination of incandescent lamps ($60\mu E.m^{-2}.S^{-1}$). Cosmids and plasmids were selected and propagated in *Escherichia coli* strain DH5α on LB medium supplemented with antibiotics at standard concentrations as described by Maniatis *et al*. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring, New York.

EXAMPLE 2

Construction of *Synechocystis* Cosmid Genomic Library

Total genomic DNA from *Synechocystis* (PCC 6803) was partially digested with *Sau*3A and fractionated on a sucrose gradient (Ausubel *et al*. [1987] *Current Protocols in Molecular Biology*, Greene Publishing Associates and Wiley Interscience, New York). Fractions containing 30 to 40 kb DNA fragments were selected and ligated into the dephosphorylated *Bam*HI site of the cosmid vector, pDUCA7 (Buikema *et al*. [1991] *J. Bacteriol.* *173*, 1879-1885). The ligated DNA was packaged *in vitro* as described by Ausubel *et al*. (1987), and packaged phage were propagated in *E. coli* DH5α containing the *Ava*I and *Eco*4711 methylase helper plasmid, pRL528 as described by Buikema *et al*. (1991). A total of 1152 colonies were isolated randomly and maintained individually in twelve 96-well microtiter plates.

EXAMPLE 3

Gain-of-Function Expression of GLA in Anabaena

Anabaena (PCC 7120), a filamentous cyanobacterium, is deficient in GLA but contains significant amounts of linoleic acid, the precursor for GLA (Figure 2; Table 2). The Synechocystis cosmid library described in Example 2 was conjugated into Anabaena (PCC 7120) to identify transconjugants that produce GLA. Anabaena cells were grown to mid-log phase in BG11N+ liquid medium and resuspended in the same medium to a final concentration of approximately $2x10^{8}$ cells per ml. A mid-log phase culture of E. coli RP4 (Burkardt et al. [1979] J. Gen. Microbiol. 114, 341-348) grown in LB containing ampicillin was washed and resuspended in fresh LB medium. Anabaena and RP4 were then mixed and spread evenly on BG11N+ plates containing 5% LB. The cosmid genomic library was replica plated onto LB plates containing 50 μg/ml kanamycin and 17.5 μg/ml chloramphenicol and was subsequently patched onto BG11N+ plates containing Anabaena and RP4. After 24 hours of incubation at 30°C, 30 μg/ml of neomycin was underlaid; and incubation at 30°C was continued until transconjugants appeared.

Individual transconjugants were isolated after conjugation and grown in 2 ml BG11N+ liquid medium with 15 μg/ml neomycin. Fatty acid methyl esters were prepared from wild type cultures and cultures containing pools of ten transconjugants as follows. Wild type and transgenic cyanobacterial

cultures were harvested by centrifugation and washed twice with distilled water. Fatty acid methyl esters were extracted from these cultures as described by Dahmer et al. (1989) J. Amer. Oil. Chem. Soc. 66, 543-548 and were analyzed by Gas Liquid Chromatography (GLC) using a Tracor-560 equipped with a hydrogen flame ionization detector and capillary column (30 m x 0.25 mm bonded FSOT Superox II, Alltech Associates Inc., IL). Retention times and co-chromatography of standards (obtained from Sigma Chemical Co.) were used for identification of fatty acids. The average fatty acid composition was determined as the ratio of peak area of each C18 fatty acid normalized to an internal standard.

Representative GLC profiles are shown in Fig. 2. C18 fatty acid methyl esters are shown. Peaks were identified by comparing the elution times with known standards of fatty acid methyl esters and were confirmed by gas chromatography-mass spectrometry. Panel A depicts GLC analysis of fatty acids of wild type Anabaena. The arrow indicates the migration time of GLA. Panel B is a GLC profile of fatty acids of transconjugants of Anabaena with pAM542+1.8F. Two GLA producing pools (of 25 pools representing 250 transconjugants) were identified that produced GLA. Individual transconjugants of each GLA positive pool were analyzed for GLA production; two independent transconjugants, AS13 and AS75, one from each pool, were identified which expressed significant levels of GLA and which contained cosmids, cSy13 and cSy75, respectively (Figure 3). The cosmids overlap

in a region approximately 7.5 kb in length. A 3.5 kb NheI fragment of cSy75 was recloned in the vector pDUCA7 and transferred to Anabaena resulting in gain-of-function expression of GLA (Table 2).

Two NheI/Hind III subfragments (1.8 and 1.7 kb) of the 3.5 kb Nhe I fragment of cSy75-3.5 were subcloned into "pBLUESCRIPT" (Stratagene) (Figure 3) for sequencing. Standard molecular biology techniques were performed as described by Maniatis et al. (1982) and Ausubel et al. (1987). Dideoxy sequencing (Sanger et al. [1977] Proc. Natl. Acad. Sci. USA 74, 5463-5467) of pBS1.8 was performed with "SEQUENASE" (United States Biochemical) on both strands by using specific oligonucleotide primers synthesized by the Advanced DNA Technologies Laboratory (Biology Department, Texas A & M University). DNA sequence analysis was done with the GCG (Madison, WI) software as described by Devereux et al. (1984) Nucleic Acids Res. 12, 387-395.

Both NheI/HindIII subfragments were transferred into a conjugal expression vector, AM542, in both forward and reverse orientations with respect to a cyanobacterial carboxylase promoter and were introduced into Anabaena by conjugation. Transconjugants containing the 1.8 kb fragment in the forward orientation (AM542-1.8F) produced significant quantities of GLA and octadecatetraenoic acid (Figure 2; Table 2). Transconjugants containing other constructs, either reverse oriented 1.8 kb fragment or forward and reverse oriented 1.7 kb fragment, did not produce detectable levels of GLA (Table 2).

Figure 2 compares the C18 fatty acid profile of an extract from wild type Anabaena (Figure 2A) with that of transgenic Anabaena containing the 1.8 kb fragment of cSy75-3.5 in the forward orientation (Figure 2B). GLC analysis of fatty acid methyl esters from AM542-1.8F revealed a peak with a retention time identical to that of authentic GLA standard. Analysis of this peak by gas chromatography-mass spectrometry (GC-MS) confirmed that it had the same mass fragmentation pattern as a GLA reference sample. Transgenic Anabaena with altered levels of polyunsaturated fatty acids were similar to wild type in growth rate and morphology.

Table 2 Composition of C18 Fatty Acids in Wild Type and Transgenic Cyanobacteria

Strain	Fatty Acid (%)					
	18:0	18:1	18:2	18.3(α)	18.3(γ)	18.4
Wild Type						
Synechocystis (sp.PCC6803)	13.6	4.5	54.5	-	27.3	-
Anabaena (sp.PCC7120)	2.9	24.8	37.1	35.2	-	-
Synechococcus (sp.PCC7942)	20.6	79.4	-	-	-	-
***Anabaena* Transconjugants**						
cSy75	3.8	24.4	22.3	9.1	27.9	12.5
cSy75-3.5	4.3	27.6	18.1	3.2	40.4	6.4
pAM542 - 1.8F	4.2	13.9	12.1	19.1	25.4	25.4
pAM542 - 1.8R	7.7	23.1	38.4	30.8	-	-
pAM542 - 1.7F	2.8	27.8	36.1	33.3	-	-
pAM542 - 1.7R	2.8	25.4	42.3	29.6	-	-
***Synechococcus* Transformants**						
pAM854	27.8	72.2	-	-	-	-
pAM854 -Δ^{12}	4.0	43.2	46.0	-	-	-
pAM854 -Δ^{6}	18.2	81.8	-	-	-	-
pAM854 -Δ^{6}&Δ^{12}	42.7	25.3	19.5	-	16.5	-

18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3(α), linolenic acid; 18:3(γ), γ-linolenic acid; 18:4, octadecatetraenoic acid

EXAMPLE 4

Transformation of Synechococcus with Δ6 and Δ12 Desaturase Genes

A third cosmid, cSy7, which contains a Δ12-desaturase gene, was isolated by screening the Synechocystis genomic library with a oligonucleotide synthesized from the published Synechocystis Δ12-desaturase gene sequence (Wada et al. [1990] Nature (London) 347, 200-203). A 1.7 kb AvaI fragment from this cosmid containing the Δ12-desaturase gene was identified and used as a probe to demonstrate that cSy13 not only contains a Δ6-desaturase gene but also a Δ12-desaturase gene (Figure 3). Genomic Southern blot analysis further showed that both the Δ6-and Δ12-desaturase genes are unique in the Synechocystis genome so that both functional genes involved in C18 fatty acid desaturation are linked closely in the Synechocystis genome.

The unicellular cyanobacterium Synechococcus (PCC 7942) is deficient in both linoleic acid and GLA(3). The Δ12 and Δ6-desaturase genes were cloned individually and together into pAM854 (Bustos et al. [1991] J. Bacteriol. 174, 7525-7533), a shuttle vector that contains sequences necessary for the integration of foreign DNA into the genome of Synechococcus (Golden et al. [1987] Methods in Enzymol. 153, 215-231). Synechococcus was transformed with these gene constructs and colonies were selected. Fatty acid methyl esters were extracted from transgenic Synechococcus and analyzed by GLC.

Table 2 shows that the principal fatty acids of wild type Synechococcus are stearic acid (18:0) and oleic acid (18:1). Synechococcus transformed with pAM854-Δ12 expressed linoleic acid (18:2) in addition to the principal fatty acids. Transformants with pAM854-Δ6 and Δ12 produced both linoleate and GLA (Table 1). These results indicated that Synechococcus containing both Δ12- and Δ6-desaturase genes has gained the capability of introducing a second double bond at the Δ12 position and a third double bond at the Δ6 position of C18 fatty acids. However, no changes in fatty acid composition was observed in the transformant containing pAM854-Δ6, indicating that in the absence of substrate synthesized by the Δ12 desaturase, the Δ6-desaturase is inactive. This experiment further confirms that the 1.8 kb NheI/HindIII fragment (Figure 3) contains both coding and promoter regions of the Synechocystis Δ6-desaturase gene. Transgenic Synechococcus with altered levels of polyunsaturated fatty acids were similar to wild type in growth rate and morphology.

EXAMPLE 5

Nucleotide Sequence of Δ6-Desaturase

The nucleotide sequence of the 1.8 kb fragment of cSy75-3.5 including the functional Δ6-desaturase gene was determined. An open reading frame encoding a polypeptide of 359 amino acids was identified (Figure 4). A Kyte-Doolittle hydropathy analysis (Kyte et al. [1982] J. Mol. Biol. 157, 105-132) identified two regions of hydrophobic amino acids that could represent transmembrane domains (Figure 1A); furthermore, the hydropathic profile of the Δ6-desaturase is similar to that of the Δ12-desaturase gene (Figure 1B; Wada et al.) and Δ9-desaturases (Thiede et al. [1986] J. Biol. Chem. 261, 13230-13235). However, the sequence similarity between the Synechocystis Δ6- and Δ12-desaturases is less than 40% at the nucleotide level and approximately 18% at the amino acid level.

EXAMPLE 6

Transfer of Cyanobacterial Δ^6-Desaturase into Tobacco

The cyanobacterial Δ^6-desaturase gene was mobilized into a plant expression vector and transferred to tobacco using Agrobacterium mediated gene transfer techniques. To ensure that the transferred desaturase is appropriately expressed in leaves and developing seeds and that the desaturase gene product is targeted to the endoplasmic reticulum or the chloroplast, various expression cassettes with Synechocystis Δ-desaturase open reading frame (ORF) were constructed. Components of these cassettes include: (i) a 35S promoter or seed specific promoter derived from the sunflower helianthinin gene to drive Δ^5-desaturase gene expression in all plant tissues or only in developing seeds respectively, (ii) a putative signal peptide either from carrot extensin gene or sunflower helianthinin gene to target newly synthesized Δ^6-desaturase into the ER, (iii) an ER lumen retention signal sequence (KDEL) at the COOH-terminal of the Δ^6-desaturase ORF, and (iv) an optimized transit peptide to target Δ^6 desaturase into the chloroplast. The 35S promoter is a derivative of pRTL2 described by Restrepo et al. (1990). The optimized transit peptide sequence is described by Van de Broeck et al. (1985). The carrot extensin signal peptide is described by Chen et al (1985) EMBO J. 9, 2145.

Transgenic tobacco plants were produced containing a chimeric cyanobacterial desaturase gene,

comprised of the <u>Synechocystis</u> Δ^5 desaturase gene fused to an endoplasmic reticulum retention sequence (KDEL) and extensin signal peptide driven by the CaMV 35S promoter. PCR amplifications of transgenic tobacco genomic DNA indicate that the Δ^6 desaturase gene was incorporated into the tobacco genome. Fatty acid methyl esters of leaves of these transgenic tobacco plants were extracted and analyzed by Gas Liquid Chromatography (GLC). These transgenic tobacco accumulated significant amounts of GLA (Figure 4). Figure 4 shows fatty acid methyl esters as determined by GLC. Peaks were identified by comparing the elution times with known standards of fatty acid methyl ester. Accordingly, cyanobacterial genes involved in fatty acid metabolism can be used to generate transgenic plants with altered fatty acid compositions.

EXAMPLE 7

Construction of Borage cDNA library

Membrane bound polysomes were isolated from borage seeds 12 days post pollination (12 DPP) using the protocol established for peas by Larkins and Davies (1975 Plant Phys. 55:749-756). RNA was extracted from the polysomes as described by Mechler (1987 Methods in Enzymology 152:241-248, Academic Press).

Poly-A+ RNA was isolated from the membrane bound polysomal RNA by use of Oligotex-dT beads (Qiagen). Corresponding cDNA was made using Stratagene's ZAP cDNA synthesis kit. The cDNA library was constructed in the lambda ZAP II vector (Stratagene) using the lambda ZAP II vector kit. The primary library was packaged in Gigapack II Gold packaging extract (Stratagene). The library was used to generate expressed sequence tags (ESTs), and sequences corresponding to the tags were used to scan the GenBank database.

EXAMPLE 8

Hybridization Protocol

Hybridization probes for screening the borage cDNA library were generated by using random primed DNA synthesis as described by Ausubel et al (1994 Current Protocols in Molecular Biology, Wiley Interscience, N.Y.) and corresponded to previously identified abundantly expressed seed storage protein cDNAs. Unincorporated nucleotides were removed by use of a G-50 spin column (Boehringer Manheim). Probe was denatured for hybridization by boiling in a water bath for 5 minutes, then quickly cooled on ice. Filters for hybridization were prehybridized at 60°C for 2-4 hours in prehybridization solution (6XSSC [Maniatis et al 1984 Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory], 1X Denharts Solution, 0.05% sodium pyrophosphate, 100 μg/ml denatured salmon sperm DNA). Denatured probe was added to the hybridization solution (6X SSC, 1X Denharts solution, 0.05% sodium pyrophosphate, 100 μg/ml denatured salmon sperm DNA) and incubated at 60°C with agitation overnight. Filters were washed in 4x, 2x, and 1x SET washes for 15 minutes each at 60°C. A 20X SET stock solution is 3M NaCl, 0.4 M Tris base, 20 mM $Na_2EDTA\text{-}2H_2O$. The 4X SET wash was 4X SET, 12.5 mM PO_4, pH 6.8 and 0.2% SDS. The 2X SET wash was 2X SET, 12.5 mM PO_4, pH 6.8 and 0.2% SDS. The 1X SET wash was 1X SET, 12.5 mM PO_4, pH 6.8 and 0.2% SDS. Filters were allowed to air dry and were then exposed to X-ray film for 24 hours with intensifying screens at -80°C.

EXAMPLE 9

Random sequencing of cDNAs from a borage seed (12 DPP) membrane-bound polysomal library

The borage cDNA library was plated at low density (500 pfu on 150 mm petri dishes). Highly prevalent seed storage protein cDNAs were "subtracted" by screening with the previously identified corresponding cDNAs. Non-hybridizing plaques were excised using Stratagene's excision protocol and reagents. Resulting bacterial colonies were used to inoculate liquid cultures and were either sequenced manually or by an ABI automated sequencer. Each cDNA was sequenced once and a sequence tag generated from 200-300 base pairs. All sequencing was performed by cycle sequencing (Epicentre). Over 300 ESTs were generated. Each sequence tag was compared to GenBank database by BLASTX computer program and a number of lipid metabolism genes, including the Δ6-desaturase were identified.

Database searches with a cDNA clone designated mbp-65 using BLASTX with the GenBank database resulted in a significant match to the Synechocystis Δ6-desaturase. It was determined however, that this clone was not a full length cDNA. A full length cDNA was isolated using mbp-65 to screen the borage membrane-bound polysomal library. The sequence of the isolated cDNA was determined (Fig. 5A, SEQ ID NO:4) and the protein sequence of the open reading frame (Fig. 5B, SEQ ID NO:5) was compared to other known desaturases using Geneworks

(IntelligGenetics) protein alignment program (Fig. 2). This alignment indicated that the cDNA was the borage Δ6-desaturase gene.

Although similar to other known plant desaturases, the borage delta 6-desaturase is distinct as indicated in the dendrogram shown in Fig. 6. Furthermore, comparison of the amino acid sequences characteristic of desaturases, particularly those proposed to be involved in metal binding (metal box 1 and metal box 2), illustrates the differences between the borage delta 6-desaturase and other plant desaturases (Table 3).

The borage delta 6-desaturase is distinguished from the cyanobacterial form not only in over all sequence (Fig. 6) but also in the lipid box, metal box 1 and metal box 2 amino acid motifs (Table 3). As Table 3 indicates, all three motifs are novel in sequence. Only the borage delta 6-desaturase metal box 2 shown some relationship to the <u>Synechocystis</u> delta-6 desaturase metal box 2.

In addition, the borage delta 6-desaturase is also distinct from another borage desaturase gene, the delta-12 desaturase. P1-81 is a full length cDNA that was identified by EST analysis and shows high similarity to the <u>Arabidopsis</u> delta-12 desaturase (Fad 2). A comparison of the lipid box, metal box 1 and metal box 2 amino acid motifs (Table 3) in borage delta 6 and delta-12 desaturases indicates that little homology exists in these regions. The placement of the two sequences in the dendrogram in Fig. 6 indicates how distantly related these two genes are.

Table 3. Comparison of common amino acid motifs in membrane-bound desaturases

Desaturase	Amino Acid Motif: Lipid Box	Metal Box 1	Metal Box 2
Borage Δ^6	WIGHDAGH (SEQ. ID. NO: 6)	HNAHH (SEQ. ID. NO: 12)	FQIEHH (SEQ. ID. NO: 20)
Synechocystis Δ^6	NVGHDANH (SEQ. ID. NO: 7)	HNYLHH (SEQ. ID. NO: 13)	HQVTHH (SEQ. ID. NO: 21)
Arab. chloroplast Δ^{15}	VLGHDCGH (SEQ. ID. NO: 8)	HRTHH (SEQ. ID. NO: 14)	HVIHH (SEQ. ID. NO: 22)
Rice Δ^{15}	VLGHDCGH (SEQ. ID. NO: 8)	HRTHH (SEQ. ID. NO: 14)	HVIHH (SEQ. ID. NO: 22)
Glycine chloroplast Δ^{15}	VLGHDCGH (SEQ. ID. NO: 8)	HRTHH (SEQ. ID. NO: 14)	HVIHH (SEQ. ID. NO: 22)
Arab. fad3 (Δ^{15})	VLGHDCGH (SEQ. ID. NO: 8)	HRTHH (SEQ. ID. NO: 14)	HVIHH (SEQ. ID. NO: 22)
Brassica fad3 (Δ^{15})	VLGHDCGH (SEQ. ID. NO: 8)	HRTHH (SEQ. ID. NO: 14)	HVIHH (SEQ. ID. NO: 22)
Borage Δ^{12} (P1-81)*	VIAHECGH (SEQ. ID. NO: 9)	HRRHH (SEQ. ID. NO: 15)	HVAHH (SEQ. ID. NO: 23)
Arab. fad2 (Δ^{17})	VIAHECGH (SEQ. ID. NO: 9)	HRRHH (SEQ. ID. NO: 15)	HVAHH (SEQ. ID. NO: 23)
Arab. chloroplast Δ^{12}	VIGHDCAH (SEQ. ID. NO: 10)	HDRHH (SEQ. ID. NO: 16)	HIPHH (SEQ. ID. NO: 24)
Glycine plastid Δ^{12}	VIGHDCAH (SEQ. ID. NO: 10)	HDRHH (SEQ. ID. NO: 16)	HIPHH (SEQ. ID. NO: 24)
Spinach plastidial n-6	VIGHDCAH (SEQ. ID. NO: 10)	HDQHH (SEQ. ID. NO: 17)	HIPHH (SEQ. ID. NO: 24)
Synechocystis Δ^{12}	VVGHDCGH (SEQ. ID. NO: 11)	HDHHH (SEQ. ID. NO: 18)	HIPHH (SEQ. ID. NO: 24)
Anabaena Δ^{12}	VLGHDCGH (SEQ. ID. NO: 8)	HNHHH (SEQ. ID. NO: 19)	HVPHH (SEQ. ID. NO: 25)

*P1-81 is a full length cDNA which was identified by EST analysis and shows high similarity to the Arbidopsis Δ12 desaturase (fad2)

EXAMPLE 10

Construction of 222.1Δ^{6}NOS for transient and expression

The vector pBI221 (Jefferson et al. 1987 EMBO J. 6:3901-3907) was prepared for ligation by digestion with BamHI and EcoICR I (Promega) which excises the GUS coding region leaving the 35S promoter and NOS terminator intact. The borage Δ 6-desaturase cDNA was excised from the Bluescript plasmid (Stratagene) by digestion with BamHI and XhoI. The XhoI end was made blunt by use of the Klenow fragment. This fragment was then cloned into the BamHI/EcoICR I sites of pBI221, yielding 221.Δ^{6}NOS (Fig. 7). In 221.Δ^6.NOS, the remaining portion (backbone) of the restriction map depicted in Fig. 7 is pBI221.

EXAMPLE 11

Construction of 121.Δ^6.NOS for stable transformation

The vector pBI121 (Jefferson et al. 1987 EMBO J. 6:3901-3907) was prepared for ligation by digestion with BamHI and EcoICR I (Promega) which excises the GUS coding region leaving the 35S promoter and NOS terminator intact. The borage Δ 6-desaturase cDNA was excised from the Bluescript plasmid (Stratagene) by digestion with BamHI and XhoI. The XhoI end was made blunt by use of the Klenow fragment. This fragment was then cloned into the BamHI/EcoICR I sites of pBI121, yielding 121.1Δ^6NOS (Fig. 7). In 121.Δ^6.NOS, the remaining portion (backbone) of the restriction map depicted in Fig. 7 is pBI121.

EXAMPLE 12

Transient Expression

All work involving protoplasts was performed in a sterile hood. One ml of packed carrot suspension cells were digested in 30 mls plasmolyzing solution (25 g/l KCl, 3.5 g/l $CaCl_2$-H_2O, 10mM MES, pH 5.6 and 0.2 M mannitol) with 1% cellulase, 0.1% pectolyase, and 0.1% dreisalase overnight, in the dark, at room temperature. Released protoplasts were filtered through a 150 μm mesh and pelleted by centrifugation (100x g, 5 min.) then washed twice in plasmolyzing solution. Protoplasts were counted using a double chambered hemocytometer. DNA was transfected into the protoplasts by PEG treatment as described by Nunberg and Thomas (1993 <u>Methods in Plant Molecular Biology and Biotechnology</u>, B.R. Glick and J.E. Thompson, eds. pp. 241-248) using 10^6 protoplasts and 50-70 ug of plasmid DNA (221.Δ6.NOS). Protoplasts were cultured in 5 mls of MS media supplemented with 0.2M mannitol and 3 μm 2,4-D for 48 hours in the dark with shaking.

EXAMPLE 13

Stable transformation of tobacco

121.Δ^6.NOS plasmid construction was used to transform tobacco (Nicotiana tabacum cv. xanthi) via Agrobacterium according to standard procedures (Horsh et al., 1985 Science 227: 1229-1231; Bogue et al., 1990 Mol. Gen. Genet. 221:49-57), except that initial transformants were selected on 100 ug/ml kanamycin.

EXAMPLE 14

Preparation and analysis of fatty acid methyl esters (FAMEs)

Tissue from transfected protoplasts and transformed tobacco plants was frozen in liquid nitrogen and lyophilized overnight. FAMEs were prepared as described by Dahmer et al (1989 J. Amer. Oil Chem. Soc. 66:543-548). In some cases, the solvent was evaporated again, and the FAMEs were resuspended in ethyl acetate and extracted once with deionized water to remove any water soluble contaminants. The FAMEs were analyzed by gas chromatography (GC) on a J&W Scientific DB-wax column (30 m length, 0.25 mm ID, 0.25 um film).

An example of a transient assay is shown in Fig. 8 which represents three independent transfections pooled together. The addition of the borage Δ6-desaturase cDNA corresponds with the appearance of gamma linolenic acid (GLA) which is one of the possible products of Δ6-desaturase.

Figures 9 and 10 depict GC profiles of the FAMES derived from leaf and seed tissue, respectively, of control and transformed tobacco plants. Figure 9A provides the profile of leaf tissue of wild-type tobacco (xanthi); Figure 9B provides the profile of leaf tissue from a tobacco plant transformed with the borage Δ-6 desaturase under the transcriptional control of the 35S CaMV promoter (pBI 121Δ^6NOS). Peaks correspond to 18:2, 18:3γ (GLA), 18:3α and 18:4 (octadecanonic acid). Figure 10A shows the GC profile of seeds of a wild-type tobacco; Figure 10B shows the

profile of seed tissue of a tobacco plant transformed with pBI 121Δ^6NOS. Peaks correspond to 18:2, 18:3γ(GLA) and 18:3α.

The relative distribution of the C_{18} fatty acids in control and transgenic tobacco seeds is shown in Table 4.

TABLE 4

Fatty Acid	Xanthi	pBI121Δ^6NOS
18:0	4.0%	2.5%
18:1	13%	13%
18:2	82%	82%
18:3γ(GLA)	-	2.7%
18:3α	0.82%	1.4%

The foregoing results demonstrate that GLA is incorporated into the triacylglycerides of transgenic tobacco leaves and seeds containing the borage Δ6-desaturase.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Rhone-Poulenc Agrochimie

(ii) TITLE OF INVENTION: PRODUCTION OF GAMMA LINOLENIC ACID BY A DELTA 6-DESATURASE

(iii) NUMBER OF SEQUENCES: 25

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Scully, Scott, Murphy & Presser
(B) STREET: 400 Garden City Plaza
(C) CITY: Garden City
(D) STATE: New York
(E) COUNTRY: United States
(F) ZIP: 11530

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE: 30-DEC-1994
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Presser, Leopold
(B) REGISTRATION NUMBER: 19,827
(C) REFERENCE/DOCKET NUMBER: 8383ZYXW

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (516) 742-4343
(B) TELEFAX: (516) 742-4366
(C) TELEX: 230 901 SANS UR

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 3588 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

(A) NAME/KEY: CDS
(B) LOCATION: 2002..3081

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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CACCTTGCCA GACCACGTTA GTTTGAGTGT TTCCGCCCTG GCGGCCCCGA TTTTTTCCTT    180
TGCGGCTTTG GGCAATCAGG CGATCGGGCA ATTGCGTTTG TTTGACCAGA CTTGGCCCAT    240
TCAGGAAATT GTCATTCACC AAGACCATCC CTGGCTCAAT TTACCCCTGG CGGATTTATG    300
GGATGATCCG AGCCGAATGT TGATCTATTA CCTACCGGCC CACAGTGAAA CGGATTTAGT    360
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ACCCCAACCC AAGACCAAAC GGCGATCGCC TTGGCGCAAA TTTTCCAAAC TGATTACCAA    480
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GGATGCCCGC CTAGAAAGAA CGTTGGCCTG CGCCAATATC AACCGAGCCG AAGCCATTGT   1020
GGTGGCCACC AGCGACGACA CCGTTAACTT GGAAATTGGC CTAACTGCCA AGGCGATCGC   1080
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AGTATTTGAA TTTGAAACGG TGCTTTGTCC GGCGGAATTG GCCACCTATT CCTTTGCGGC   1200
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GGAATTATTG GGTACCCATC TCGACTCTGG AGACGTGTTG TATTTAACCA TGCCCGCCAC   1440
TGCCCTAGAG CAACTTTGGC GATCGCCCCG TGCCACTGCT GATCCTCTGG ACTCTTTTTT   1500
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AAGCTCAAAA AGTAGCAAAA TAAGTTTAAT TCATAACTGA GTTTTACTGC TAAACAGCGG 1680

TGCAAAAAAG TCAGATAAAA TAAAAGCTTC ACTTCGGTTT TATATTGTGA CCATGGTTCC 1740

CAGGCATCTG CTCTAGGGAG TTTTTCCGCT GCCTTTAGAG AGTATTTTCT CCAAGTCGGC 1800

TAACTCCCCC ATTTTTAGGC AAAATCATAT ACAGACTATC CCAATATTGC CAGAGCTTTG 1860

ATGACTCACT GTAGAAGGCA GACTAAAATT CTAGCAATGG ACTCCCAGTT GGAATAAATT 1920

TTTAGTCTCC CCCGGCGCTG GAGTTTTTTT GTAGTTAATG GCGGTATAAT GTGAAAGTTT 1980

TTTATCTATT TAAATTTATA A ATG CTA ACA GCG GAA AGA ATT AAA TTT ACC 2031
Met Leu Thr Ala Glu Arg Ile Lys Phe Thr
1 5 10

CAG AAA CGG GGG TTT CGT CGG GTA CTA AAC CAA CGG GTG GAT GCC TAC 2079
Gln Lys Arg Gly Phe Arg Arg Val Leu Asn Gln Arg Val Asp Ala Tyr
15 20 25

TTT GCC GAG CAT GGC CTG ACC CAA AGG GAT AAT CCC TCC ATG TAT CTG 2127
Phe Ala Glu His Gly Leu Thr Gln Arg Asp Asn Pro Ser Met Tyr Leu
30 35 40

AAA ACC CTG ATT ATT GTG CTC TGG TTG TTT TCC GCT TGG GCC TTT GTG 2175
Lys Thr Leu Ile Ile Val Leu Trp Leu Phe Ser Ala Trp Ala Phe Val
45 50 55

CTT TTT GCT CCA GTT ATT TTT CCG GTG CGC CTA CTG GGT TGT ATG GTT 2223
Leu Phe Ala Pro Val Ile Phe Pro Val Arg Leu Leu Gly Cys Met Val
60 65 70

TTG GCG ATC GCC TTG GCG GCC TTT TCC TTC AAT GTC GGC CAC GAT GCC 2271
Leu Ala Ile Ala Leu Ala Ala Phe Ser Phe Asn Val Gly His Asp Ala
75 80 85 90

AAC CAC AAT GCC TAT TCC TCC AAT CCC CAC ATC AAC CGG GTT CTG GGC 2319
Asn His Asn Ala Tyr Ser Ser Asn Pro His Ile Asn Arg Val Leu Gly
95 100 105

ATG ACC TAC GAT TTT GTC GGG TTA TCT AGT TTT CTT TGG CGC TAT CGC 2367
Met Thr Tyr Asp Phe Val Gly Leu Ser Ser Phe Leu Trp Arg Tyr Arg
110 115 120

CAC AAC TAT TTG CAC CAC ACC TAC ACC AAT ATT CTT GGC CAT GAC GTG 2415
His Asn Tyr Leu His His Thr Tyr Thr Asn Ile Leu Gly His Asp Val
125 130 135

GAA ATC CAT GGA GAT GGC GCA GTA CGT ATG AGT CCT GAA CAA GAA CAT 2463
Glu Ile His Gly Asp Gly Ala Val Arg Met Ser Pro Glu Gln Glu His
140 145 150

GTT GGT ATT TAT CGT TTC CAG CAA TTT TAT ATT TGG GGT TTA TAT CTT 2511
Val Gly Ile Tyr Arg Phe Gln Gln Phe Tyr Ile Trp Gly Leu Tyr Leu
155 160 165 170

TTC ATT CCC TTT TAT TGG TTT CTC TAC GAT GTC TAC CTA GTG CTT AAT 2559
Phe Ile Pro Phe Tyr Trp Phe Leu Tyr Asp Val Tyr Leu Val Leu Asn
175 180 185

AAA GGC AAA TAT CAC GAC CAT AAA ATT CCT CCT TTC CAG CCC CTA GAA 2607
Lys Gly Lys Tyr His Asp His Lys Ile Pro Pro Phe Gln Pro Leu Glu
190 195 200

TTA GCT AGT TTG CTA GGG ATT AAG CTA TTA TGG CTC GGC TAC GTT TTC 2655
Leu Ala Ser Leu Leu Gly Ile Lys Leu Leu Trp Leu Gly Tyr Val Phe
205 210 215

GGC TTA CCT CTG GCT CTG GGC TTT TCC ATT CCT GAA GTA TTA ATT GGT 2703
Gly Leu Pro Leu Ala Leu Gly Phe Ser Ile Pro Glu Val Leu Ile Gly
220 225 230

GCT TCG GTA ACC TAT ATG ACC TAT GGC ATC GTG GTT TGC ACC ATC TTT 2751
Ala Ser Val Thr Tyr Met Thr Tyr Gly Ile Val Val Cys Thr Ile Phe
235 240 245 250

ATG CTG GCC CAT GTG TTG GAA TCA ACT GAA TTT CTC ACC CCC GAT GGT 2799
Met Leu Ala His Val Leu Glu Ser Thr Glu Phe Leu Thr Pro Asp Gly
255 260 265

GAA TCC GGT GCC ATT GAT GAC GAG TGG GCT ATT TGC CAA ATT CGT ACC 2847
Glu Ser Gly Ala Ile Asp Asp Glu Trp Ala Ile Cys Gln Ile Arg Thr
270 275 280

ACG GCC AAT TTT GCC ACC AAT AAT CCC TTT TGG AAC TGG TTT TGT GGC 2895
Thr Ala Asn Phe Ala Thr Asn Asn Pro Phe Trp Asn Trp Phe Cys Gly
285 290 295

GGT TTA AAT CAC CAA GTT ACC CAC CAT CTT TTC CCC AAT ATT TGT CAT 2943
Gly Leu Asn His Gln Val Thr His His Leu Phe Pro Asn Ile Cys His
300 305 310

ATT CAC TAT CCC CAA TTG GAA AAT ATT ATT AAG GAT GTT TGC CAA GAG 2991
Ile His Tyr Pro Gln Leu Glu Asn Ile Ile Lys Asp Val Cys Gln Glu
315 320 325 330

TTT GGT GTG GAA TAT AAA GTT TAT CCC ACC TTC AAA GCG GCG ATC GCC 3039
Phe Gly Val Glu Tyr Lys Val Tyr Pro Thr Phe Lys Ala Ala Ile Ala
335 340 345

TCT AAC TAT CGC TGG CTA GAG GCC ATG GGC AAA GCA TCG TGACATTGCC 3088
Ser Asn Tyr Arg Trp Leu Glu Ala Met Gly Lys Ala Ser
350 355 360

TTGGGATTGA AGCAAAATGG CAAAATCCCT CGTAAATCTA TGATCGAAGC CTTTCTGTTG 3148

CCCGCCGACC AAATCCCCGA TGCTGACCAA AGGTTGATGT TGGCATTGCT CCAAACCCAC 3208

TTTGAGGGGG TTCATTGGCC GCAGTTTCAA GCTGACCTAG GAGGCAAAGA TTGGGTGATT 3268

TTGCTCAAAT CCGCTGGGAT ATTGAAAGGC TTCACCACCT TTGGTTTCTA CCCTGCTCAA 3328

TGGGAAGGAC AAACCGTCAG AATTGTTTAT TCTGGTGACA CCATCACCGA CCCATCCATG 3388

TGGTCTAACC CAGCCCTGGC CAAGGCTTGG ACCAAGGCCA TGCAAATTCT CCACGAGGCT 3448

AGGCCAGAAA AATTATATTG GCTCCTGATT TCTTCCGGCT ATCGCACCTA CCGATTTTTG 3508

AGCATTTTTG CCAAGGAATT CTATCCCCAC TATCTCCATC CCACTCCCCC GCCTGTACAA 3568

AATTTTATCC ATCAGCTAGC 3588

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 359 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Leu Thr Ala Glu Arg Ile Lys Phe Thr Gln Lys Arg Gly Phe Arg
1 5 10 15

Arg Val Leu Asn Gln Arg Val Asp Ala Tyr Phe Ala Glu His Gly Leu
20 25 30

Thr Gln Arg Asp Asn Pro Ser Met Tyr Leu Lys Thr Leu Ile Ile Val
35 40 45

Leu Trp Leu Phe Ser Ala Trp Ala Phe Val Leu Phe Ala Pro Val Ile
50 55 60

Phe Pro Val Arg Leu Leu Gly Cys Met Val Leu Ala Ile Ala Leu Ala
65 70 75 80

Ala Phe Ser Phe Asn Val Gly His Asp Ala Asn His Asn Ala Tyr Ser
85 90 95

Ser Asn Pro His Ile Asn Arg Val Leu Gly Met Thr Tyr Asp Phe Val
100 105 110

Gly Leu Ser Ser Phe Leu Trp Arg Tyr Arg His Asn Tyr Leu His His
115 120 125

Thr Tyr Thr Asn Ile Leu Gly His Asp Val Glu Ile His Gly Asp Gly
130 135 140

Ala Val Arg Met Ser Pro Glu Gln Glu His Val Gly Ile Tyr Arg Phe
145 150 155 160

```
Gln Gln Phe Tyr Ile Trp Gly Leu Tyr Leu Phe Ile Pro Phe Tyr Trp
                165                 170                 175

Phe Leu Tyr Asp Val Tyr Leu Val Leu Asn Lys Gly Lys Tyr His Asp
            180                 185                 190

His Lys Ile Pro Pro Phe Gln Pro Leu Glu Leu Ala Ser Leu Leu Gly
        195                 200                 205

Ile Lys Leu Leu Trp Leu Gly Tyr Val Phe Gly Leu Pro Leu Ala Leu
    210                 215                 220

Gly Phe Ser Ile Pro Glu Val Leu Ile Gly Ala Ser Val Thr Tyr Met
225                 230                 235                 240

Thr Tyr Gly Ile Val Val Cys Thr Ile Phe Met Leu Ala His Val Leu
                245                 250                 255

Glu Ser Thr Glu Phe Leu Thr Pro Asp Gly Glu Ser Gly Ala Ile Asp
            260                 265                 270

Asp Glu Trp Ala Ile Cys Gln Ile Arg Thr Thr Ala Asn Phe Ala Thr
        275                 280                 285

Asn Asn Pro Phe Trp Asn Trp Phe Cys Gly Gly Leu Asn His Gln Val
    290                 295                 300

Thr His His Leu Phe Pro Asn Ile Cys His Ile His Tyr Pro Gln Leu
305                 310                 315                 320

Glu Asn Ile Ile Lys Asp Val Cys Gln Glu Phe Gly Val Glu Tyr Lys
                325                 330                 335

Val Tyr Pro Thr Phe Lys Ala Ala Ile Ala Ser Asn Tyr Arg Trp Leu
            340                 345                 350

Glu Ala Met Gly Lys Ala Ser
        355
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1884 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
AGCTTCACTT CGGTTTTATA TTGTGACCAT GGTTCCCAGG CATCTGCTCT AGGGAGTTTT      60

TCCGCTGCCT TTAGAGAGTA TTTTCTCCAA GTCGGCTAAC TCCCCCATTT TTAGGCAAAA     120
```

TCATATACAG ACTATCCCAA TATTGCCAGA GCTTTGATGA CTCACTGTAG AAGGCAGACT 180
AAAATTCTAG CAATGGACTC CCAGTTGGAA TAAATTTTTA GTCTCCCCCG GCGCTGGAGT 240
TTTTTTGTAG TTAATGGCGG TATAATGTGA AAGTTTTTTA TCTATTTAAA TTTATAAATG 300
CTAACAGCGG AAAGAATTAA ATTTACCCAG AAACGGGGGT TTCGTCGGGT ACTAAACCAA 360
CGGGTGGATG CCTACTTTGC CGAGCATGGC CTGACCCAAA GGGATAATCC CTCCATGTAT 420
CTGAAAACCC TGATTATTGT GCTCTGGTTG TTTTCCGCTT GGGCCTTTGT GCTTTTTGCT 480
CCAGTTATTT TTCCGGTGCG CCTACTGGGT TGTATGGTTT TGGCGATCGC CTTGGCGGCC 540
TTTTCCTTCA ATGTCGGCCA CGATGCCAAC CACAATGCCT ATTCCTCCAA TCCCCACATC 600
AACCGGGTTC TGGGCATGAC CTACGATTTT GTCGGGTTAT CTAGTTTTCT TTGGCGCTAT 660
CGCCACAACT ATTTGCACCA CACCTACACC AATATTCTTG GCCATGACGT GGAAATCCAT 720
GGAGATGGCG CAGTACGTAT GAGTCCTGAA CAAGAACATG TTGGTATTTA TCGTTTCCAG 780
CAATTTTATA TTTGGGGTTT ATATCTTTTC ATTCCCTTTT ATTGGTTTCT CTACGATGTC 840
TACCTAGTGC TTAATAAAGG CAAATATCAC GACCATAAAA TTCCTCCTTT CCAGCCCCTA 900
GAATTAGCTA GTTTGCTAGG GATTAAGCTA TTATGGCTCG GCTACGTTTT CGGCTTACCT 960
CTGGCTCTGG GCTTTTCCAT TCCTGAAGTA TTAATTGGTG CTTCGGTAAC CTATATGACC 1020
TATGGCATCG TGGTTTGCAC CATCTTTATG CTGGCCCATG TGTTGGAATC AACTGAATTT 1080
CTCACCCCCG ATGGTGAATC CGGTGCCATT GATGACGAGT GGGCTATTTG CCAAATTCGT 1140
ACCACGGCCA ATTTTGCCAC CAATAATCCC TTTTGGAACT GGTTTTGTGG CGGTTTAAAT 1200
CACCAAGTTA CCCACCATCT TTTCCCCAAT ATTTGTCATA TTCACTATCC CCAATTGGAA 1260
AATATTATTA AGGATGTTTG CCAAGAGTTT GGTGTGGAAT ATAAAGTTTA TCCCACCTTC 1320
AAAGCGGCGA TCGCCTCTAA CTATCGCTGG CTAGAGGCCA TGGGCAAAGC ATCGTGACAT 1380
TGCCTTGGGA TTGAAGCAAA ATGGCAAAAT CCCTCGTAAA TCTATGATCG AAGCCTTTCT 1440
GTTGCCCGCC GACCAAATCC CCGATGCTGA CCAAAGGTTG ATGTTGGCAT TGCTCCAAAC 1500
CCACTTTGAG GGGGTTCATT GGCCGCAGTT TCAAGCTGAC CTAGGAGGCA AAGATTGGGT 1560
GATTTTGCTC AAATCCGCTG GGATATTGAA AGGCTTCACC ACCTTTGGTT TCTACCCTGC 1620
TCAATGGGAA GGACAAACCG TCAGAATTGT TTATTCTGGT GACACCATCA CCGACCCATC 1680
CATGTGGTCT AACCCAGCCC TGGCCAAGGC TTGGACCAAG GCCATGCAAA TTCTCCACGA 1740
GGCTAGGCCA GAAAAATTAT ATTGGCTCCT GATTTCTTCC GGCTATCGCA CCTACCGATT 1800

TTTGAGCATT TTTGCCAAGG AATTCTATCC CCACTATCTC CATCCCACTC CCCCGCCTGT 1860

ACAAAATTTT ATCCATCAGC TAGC 1884

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1685 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AATATCTGCC TACCCTCCCA AAGAGAGTAG TCATTTTTCA TCAATGGCTG CTCAAATCAA 60

GAAATACATT ACCTCAGATG AACTCAAGAA CCACGATAAA CCCGGAGATC TATGGATCTC 120

GATTCAAGGG AAAGCCTATG ATGTTTCGGA TTGGGTGAAA GACCATCCAG GTGGCAGCTT 180

TCCCTTGAAG AGTCTTGCTG GTCAAGAGGT AACTGATGCA TTTGTTGCAT TCCATCCTGC 240

CTCTACATGG AAGAATCTTG ATAAGTTTTT CACTGGGTAT TATCTTAAAG ATTACTCTGT 300

TTCTGAGGTT TCTAAAGATT ATAGGAAGCT TGTGTTTGAG TTTTCTAAAA TGGGTTTGTA 360

TGACAAAAAA GGTCATATTA TGTTTGCAAC TTTGTGCTTT ATAGCAATGC TGTTTGCTAT 420

GAGTGTTTAT GGGGTTTTGT TTTGTGAGGG TGTTTTGGTA CATTTGTTTT CTGGGTGTTT 480

GATGGGGTTT CTTTGGATTC AGAGTGGTTG GATTGGACAT GATGCTGGGC ATTATATGGT 540

AGTGTCTGAT TCAAGGCTTA ATAAGTTTAT GGGTATTTTT GCTGCAAATT GTCTTTCAGG 600

AATAAGTATT GGTTGGTGGA AATGGAACCA TAATGCACAT CACATTGCCT GTAATAGCCT 660

TGAATATGAC CCTGATTTAC AATATATACC ATTCCTTGTT GTGTCTTCCA AGTTTTTTGG 720

TTCACTCACC TCTCATTTCT ATGAGAAAAG GTTGACTTTT GACTCTTTAT CAAGATTCTT 780

TGTAAGTTAT CAACATTGGA CATTTTACCC TATTATGTGT GCTGCTAGGC TCAATATGTA 840

TGTACAATCT CTCATAATGT TGTTGACCAA GAGAAATGTG TCCTATCGAG CTCAGGAACT 900

CTTGGGATGC CTAGTGTTCT CGATTTGGTA CCCGTTGCTT GTTTCTTGTT TGCCTAATTG 960

GGGTGAAAGA ATTATGTTTG TTATTGCAAG TTTATCAGTG ACTGGAATGC AACAAGTTCA 1020

GTTCTCCTTG AACCACTTCT CTTCAAGTGT TTATGTTGGA AAGCCTAAAG GGAATAATTG 1080

GTTTGAGAAA CAAACGGATG GGACACTTGA CATTTCTTGT CCTCCTTGGA TGGATTGGTT 1140

TCATGGTGGA TTGCAATTCC AAATTGAGCA TCATTTGTTT CCCAAGATGC CTAGATGCAA 1200

CCTTAGGAAA ATCTCGCCCT ACGTGATCGA GTTATGCAAG AAACATAATT TGCCTTACAA 1260

TTATGCATCT TTCTCCAAGG CCAATGAAAT GACACTCAGA ACATTGAGGA ACACAGCATT 1320

GCAGGCTAGG GATATAACCA AGCCGCTCCC GAAGAATTTG GTATGGGAAG CTCTTCACAC 1380

TCATGGTTAA AATTACCCTT AGTTCATGTA ATAATTTGAG ATTATGTATC TCCTATGTTT 1440

GTGTCTTGTC TTGGTTCTAC TTGTTGGAGT CATTGCAACT TGTCTTTTAT GGTTTATTAG 1500

ATGTTTTTTA ATATATTTTA GAGGTTTTGC TTTCATCTCC ATTATTGATG AATAAGGAGT 1560

TGCATATTGT CAATTGTTGT GCTCAATATC TGATATTTTG GAATGTACTT TGTACCACTG 1620

TGTTTTCAGT TGAAGCTCAT GTGTACTTCT ATAGACTTTG TTTAAATGGT TATGTCATGT 1680

TATTT 1685

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 448 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Met Ala Ala Gln Ile Lys Lys Tyr Ile Thr Ser Asp Glu Leu Lys Asn
1 5 10 15

His Asp Lys Pro Gly Asp Leu Trp Ile Ser Ile Gln Gly Lys Ala Tyr
20 25 30

Asp Val Ser Asp Trp Val Lys Asp His Pro Gly Gly Ser Phe Pro Leu
35 40 45

Lys Ser Leu Ala Gly Gln Glu Val Thr Asp Ala Phe Val Ala Phe His
50 55 60

Pro Ala Ser Thr Trp Lys Asn Leu Asp Lys Phe Phe Thr Gly Tyr Tyr
65 70 75 80

Leu Lys Asp Tyr Ser Val Ser Glu Val Ser Lys Asp Tyr Arg Lys Leu
85 90 95

Val Phe Glu Phe Ser Lys Met Gly Leu Tyr Asp Lys Lys Gly His Ile
100 105 110

Met Phe Ala Thr Leu Cys Phe Ile Ala Met Leu Phe Ala Met Ser Val
115 120 125

Tyr Gly Val Leu Phe Cys Glu Gly Val Leu Val His Leu Phe Ser Gly
130 135 140

Cys Leu Met Gly Phe Leu Trp Ile Gln Ser Gly Trp Ile Gly His Asp
145 150 155 160

Ala Gly His Tyr Met Val Val Ser Asp Ser Arg Leu Asn Lys Phe Met
165 170 175

Gly Ile Phe Ala Ala Asn Cys Leu Ser Gly Ile Ser Ile Gly Trp Trp
180 185 190

Lys Trp Asn His Asn Ala His His Ile Ala Cys Asn Ser Leu Glu Tyr
195 200 205

Asp Pro Asp Leu Gln Tyr Ile Pro Phe Leu Val Val Ser Ser Lys Phe
210 215 220

Phe Gly Ser Leu Thr Ser His Phe Tyr Glu Lys Arg Leu Thr Phe Asp
225 230 235 240

Ser Leu Ser Arg Phe Phe Val Ser Tyr Gln His Trp Thr Phe Tyr Pro
245 250 255

Ile Met Cys Ala Ala Arg Leu Asn Met Tyr Val Gln Ser Leu Ile Met
260 265 270

Leu Leu Thr Lys Arg Asn Val Ser Tyr Arg Ala Gln Glu Leu Leu Gly
275 280 285

Cys Leu Val Phe Ser Ile Trp Tyr Pro Leu Leu Val Ser Cys Leu Pro
290 295 300

Asn Trp Gly Glu Arg Ile Met Phe Val Ile Ala Ser Leu Ser Val Thr
305 310 315 320

Gly Met Gln Gln Val Gln Phe Ser Leu Asn His Phe Ser Ser Ser Val
325 330 335

Tyr Val Gly Lys Pro Lys Gly Asn Asn Trp Phe Glu Lys Gln Thr Asp
340 345 350

Gly Thr Leu Asp Ile Ser Cys Pro Pro Trp Met Asp Trp Phe His Gly
355 360 365

Gly Ser Gln Phe Gln Ile Glu His His Leu Phe Pro Lys Met Pro Arg
370 375 380

Cys Asn Leu Arg Lys Ile Ser Pro Tyr Val Ile Glu Leu Cys Lys Lys
385 390 395 400

His Asn Leu Pro Tyr Asn Tyr Ala Ser Phe Ser Lys Ala Asn Glu Met
405 410 415

Thr Leu Arg Thr Leu Arg Asn Thr Ala Leu Gln Ala Arg Asp Ile Thr
420 425 430

Lys Pro Leu Pro Lys Asn Leu Val Trp Glu Ala Leu His Thr His Gly
435 440 445

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Trp Ile Gly His Asp Ala Gly His
1               5
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
Asn Val Gly His Asp Ala Asn His
1               5
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
Val Leu Gly His Asp Cys Gly His
1               5
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Val Ile Ala His Glu Cys Gly His
1 5

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Val Ile Gly His Asp Cys Ala His
1 5

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Val Val Gly His Asp Cys Gly His
1 5

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

His Asn Ala His His
1 5

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
His Asn Tyr Leu His His
1               5
```

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```
His Arg Thr His His
1               5
```

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
His Arg Arg His His
1               5
```

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

His Asp Arg His His
1 5

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

His Asp Gln His His
1 5

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

His Asp His His His
1 5

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

His Asn His His His
1 5

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Phe Gln Ile Glu His His
1 5

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

His Gln Val Thr His His
1 5

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

His Val Ile His His
1 5

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids
(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

His Val Ala His His
1 5

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

His Ile Pro His His
1 5

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

His Val Pro His His
1 5

WHAT IS CLAIMED:

1. An isolated nucleic acid encoding a borage Δ6-desaturase.

2. The isolated nucleic acid of Claim 1 comprising the nucleotide sequence of SEQ ID NO: 4.

3. An isolated nucleic acid that codes for the amino acid sequence of SEQ ID NO: 5.

4. A vector comprising the nucleic acid of any one Claims 1-3.

5. An expression vector comprising the isolated nucleic acid of any one of Claims 1-3 operably linked to a promoter and optionally a termination signal capable of effecting expression of the gene product of said isolated nucleic acid.

6. The expression vector of Claim 5 wherein said promoter is a Δ-6 desaturase promoter, an *Anabaena* carboxylase promoter, a helianthinin promoter, a glycinin promoter, a napin promoter, the 35S promoter from CaMV, or a helianthinin tissue-specific promoter.

7. The expression vector of Claim 5 wherein said promoter is constitutive or tissue-specific.

8. The expression vector of Claim 5 wherein said termination signal is a *Synechocystis* termination

signal, a nopaline synthase termination signal, or a seed termination signal.

9. A cell comprising the vector of any one of Claims 4-8.

10. The cell of Claim 9 wherein said cell is an animal cell, a bacterial cell, a plant cell or a fungal cell.

11. A transgenic organism comprising the isolated nucleic acid of any one of Claims 1-3.

12. A transgenic organism comprising the vector of any one of Claims 4-8.

13. The transgenic organism of Claim 11 or 12 wherein said organism is a bacterium, a fungus, a plant or an animal.

14. A plant or progeny of said plant which has been regenerated from the plant cell of Claim 10.

15. The plant of Claim 14 wherein said plant is a sunflower, soybean, maize, tobacco, peanut, carrot or oil seed rape plant.

16. A method of producing a plant with increased gamma linolenic acid (GLA) content which comprises:

(a) transforming a plant cell with the isolated nucleic acid of any one of Claims 1-3; and

(b) regenerating a plant with increased GLA content from said plant cell.

17. A method of producing a plant with increased gamma linolenic acid (GLA) content which comprises:

(a) transforming a plant cell with the vector of any one of Claims 4-8; and

(b) regenerating a plant with increased GLA content from said plant cell.

18. The method of Claim 16 or 17 wherein said plant is a sunflower, soybean, maize, tobacco, peanut, carrot or oil seed rape plant.

19. A method of inducing production of gamma linolenic acid (GLA) in an organism deficient or lacking in GLA which comprises transforming said organism with the isolated nucleic acid of any one of Claims 1-3.

20. A method of inducing production of gamma linolenic acid (GLA) in an organism deficient or lacking in GLA which comprises transforming said organism with the vector of any one of Claims 4-8.

21. A method of inducing production of gamma linolenic acid (GLA) in an organism deficient or lacking in GLA and linoleic acid (LA) which comprises transforming said organism with an isolated nucleic acid encoding

borage Δ6-desaturase and an isolated nucleic acid encoding Δ12-desaturase.

22. The method of Claim 21 wherein said isolated nucleic acid encoding Δ6-desaturase comprises nucleotides 44 to 1390 of SEQ. ID NO: 4.

23. A method of inducing production of octadecatetraeonic acid in an organism deficient or lacking in gamma linolenic acid which comprises transforming said organism with the isolated nucleic acid of any one of Claims 1-3.

24. A method of inducing production of octadecatetraeonic acid in an organism deficient or lacking in gamma linolenic acid which comprises transforming said organism with the vector of any one of Claims 4-8.

25. The method of Claim 23 or 24 wherein said organism is a bacterium, a fungus, a plant or an animal.

26. A method of producing a plant with improved chilling resistance which comprises:

(a) transforming a plant cell with the isolated nucleic acid of any one of Claims 1-3; and

(b) regenerating said plant with improved chilling resistance from said transformed plant cell.

27. A method of producing a plant with improved chilling resistance which comprises:

(a) transforming a plant cell with the vector of any one of Claims 4-8; and

(b) regenerating said plant with improved chilling resistance from said transformed plant cell.

28. The method of Claim 26 or 27 wherein said plant is a sunflower, soybean, maize, tobacco, peanut, carrot or oil seed rape plant.

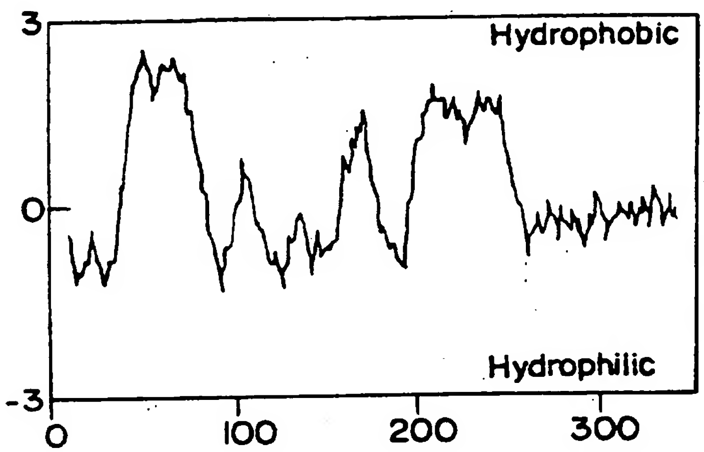

3
Hydrophobic
0
Hydrophilic
-3
0
100
200
300

FIG. 1A

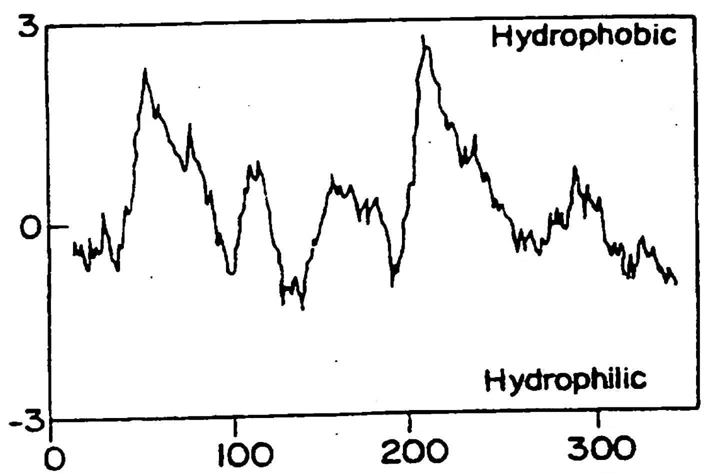

3
Hydrophobic
0
Hydrophilic
-3
0
100
200
300

FIG. 1B

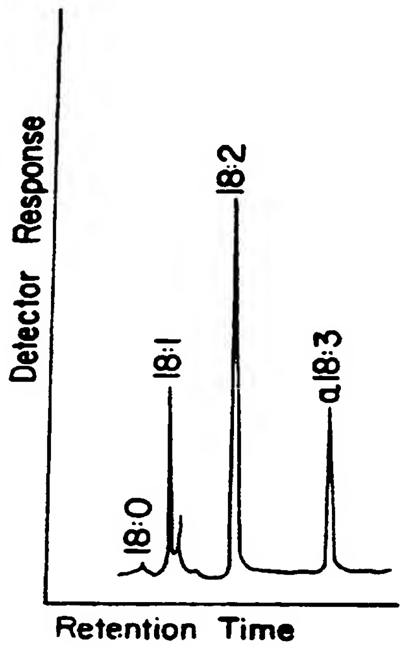


FIG. 2A

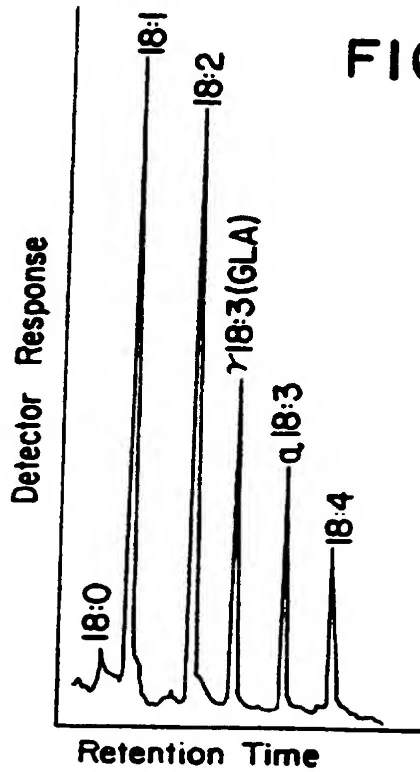


FIG. 2B

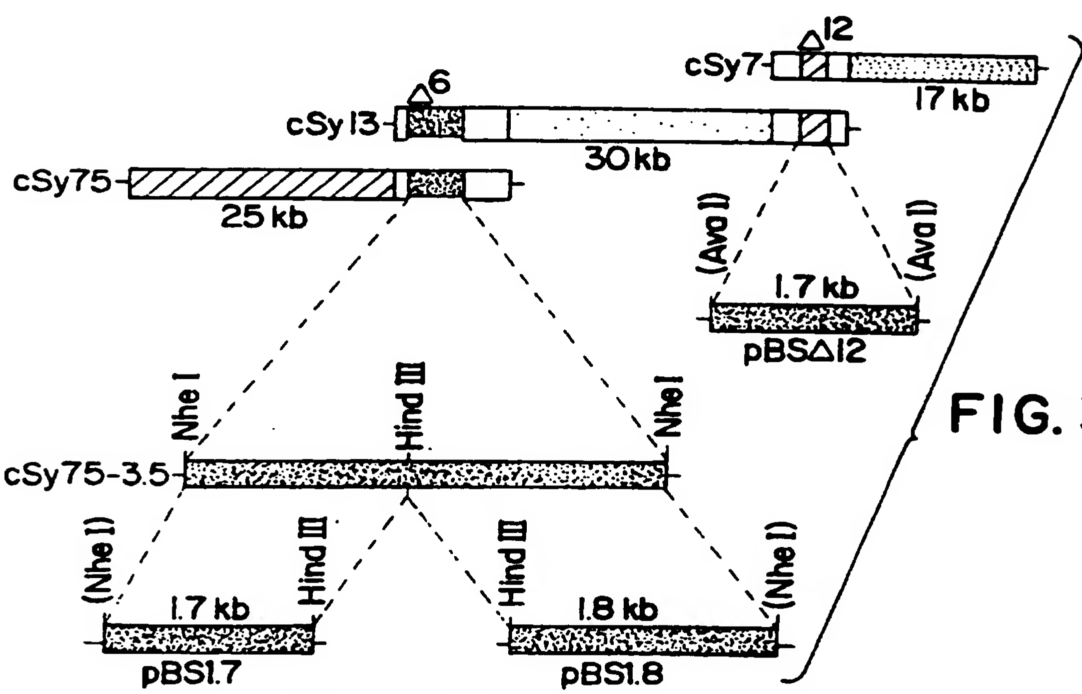


FIG. 3

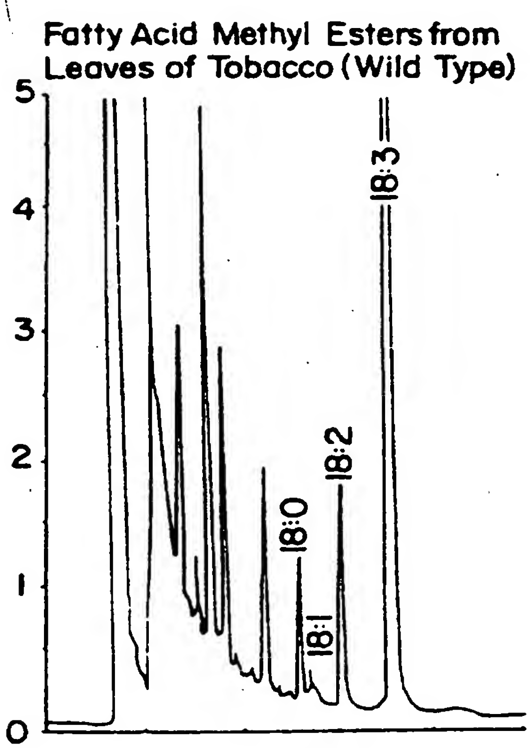

Fatty Acid Methyl Esters from
Leaves of Tobacco (Wild Type)
5
4
3
2
1
0
18:0
18:1
18:2
18:3

FIG. 4A

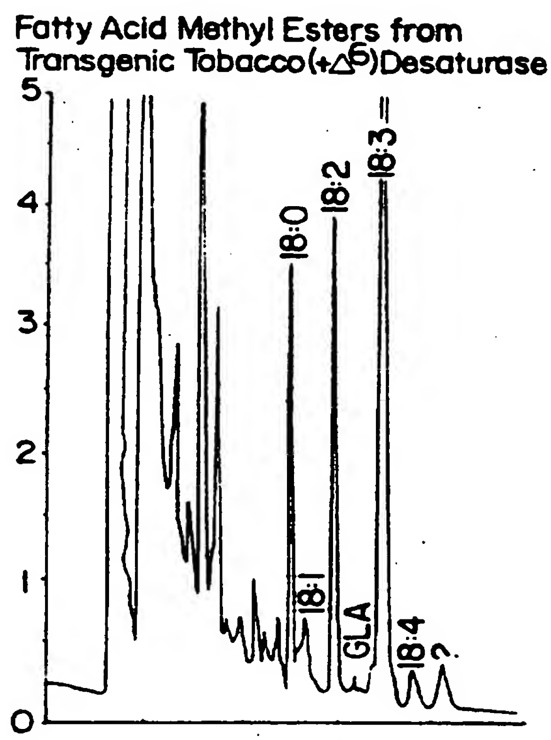

Fatty Acid Methyl Esters from
Transgenic Tobacco (+Δ6) Desaturase
5
4
3
2
1
0
18:0
18:1
18:2
GLA
18:3
18:4
?

FIG. 4B

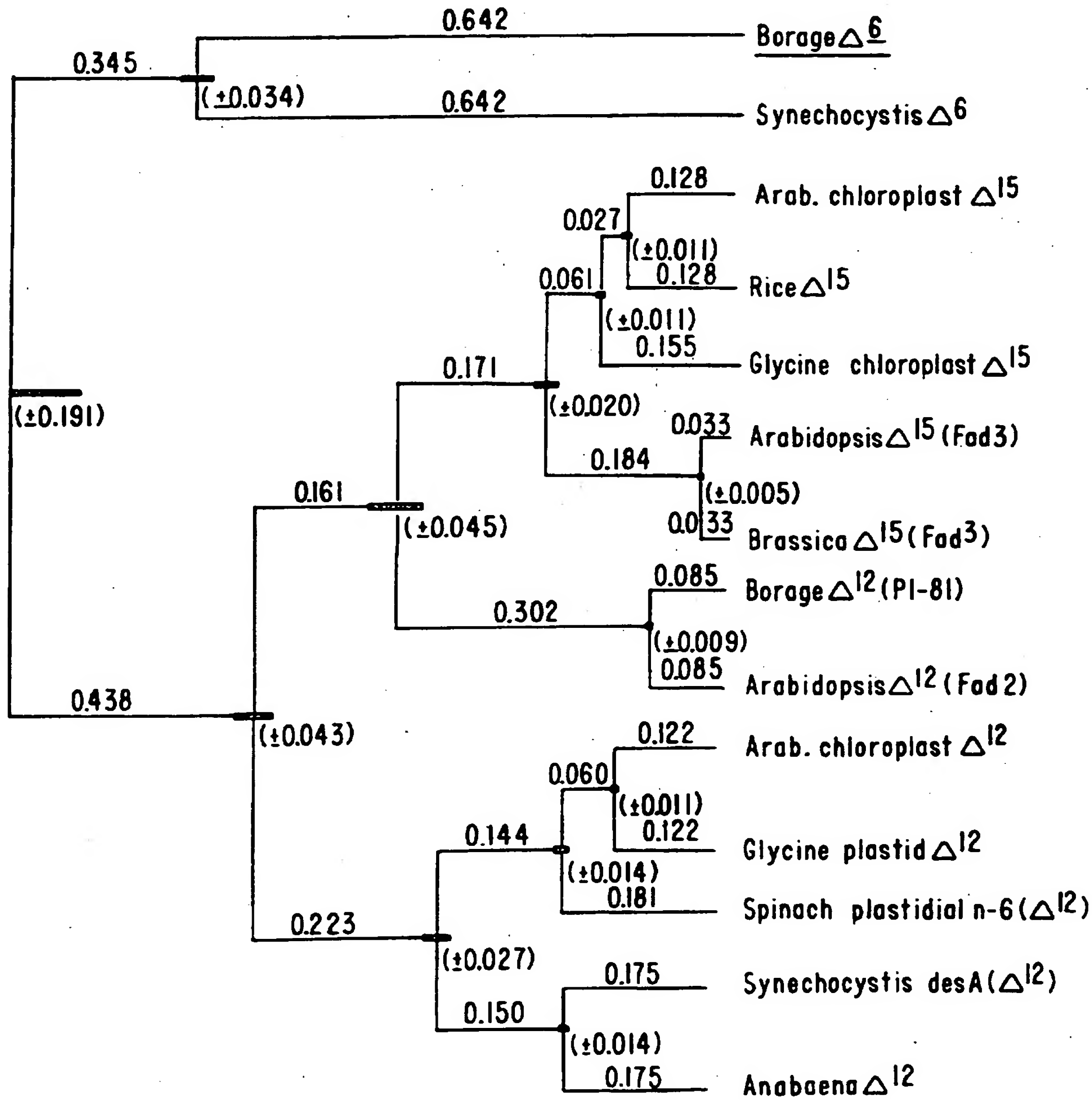
FIG.5A

```
   1 aatatctgcc taccctccca aagagagtag tcatttttca tcaatggctg ctcaaatcaa gaaatacatt acctcagatg 80
  81 aactcaagaa ccacgataaa cccggagatc tatggatctc gattcaaggg aaagcctatg atgtttcgga ttgggtgaaa 160
 161 gaccatccag gtggcagctt tcccttgaag agtcttgctg gtcaagaggt aactgatgca tttgttgcat tccatcctgc 240
 241 ctctacatgg aagaatcttg ataagttttt cactgggtat tatcttaaag attactctgt ttctgaggtt tctaaagatt 320
 321 ataggaagct tgtgtttgag ttttctaaaa tgggtttgta tgacaaaaaa ggtcatatta tgtttgcaac tttgtgcttt 400
 401 atagcaatgc tgtttgctat gagtgtttat ggggttttgt tttgtgaggg tgttttggta catttgtttt ctgggtgttt 480
 481 gatggggttt ctttggattc agagtggttg gattggacat gatgctgggc attatatggt agtgtctgat tcaaggctta 560
 561 ataagtttat gggtattttt gctgcaaatt gtctttcagg aataagtatt ggttggtgga aatggaacca taatgcacat 640
 641 cacattgcct gtaatagcct tgaatatgac cctgatttac aatatatacc attccttgtt gtgtcttcca agtttttttgg 720
 721 ttcactcacc tctcatttct atgagaaaag gttgactttt gactctttat caagattctt tgtaagttat caacattgga 800
 801 cattttaccc tattatgtgt gctgctaggc tcaatatgta tgtacaatct ctcataatgt tgttgaccaa gagaaatgtg 880
 881 tcctatcgag ctcaggaact cttgggatgc ctagtgttct cgatttggta cccgttgctt gtttcttgtt tgcctaattg 960
 961 gggtgaaaga attatgtttg ttattgcaag tttatcagtg actggaatgc aacaagttca gttctccttg aaccacttct 1040
1041 cttcaagtgt ttatgttgga aagcctaaag ggaataattg gtttgagaaa caaacggatg ggacacttga catttcttgt 1120
1121 cctccttgga tggattggtt tcatggtgga ttgcaattcc aaattgagca tcatttgttt cccaagatgc ctagatgcaa 1200
1201 ccttaggaaa atctcgccct acgtgatcga gttatgcaag aaacataatt tgccttacaa ttatgcatct ttctccaagg 1280
1281 ccaatgaaat gacactcaga acattgagga acacagcatt gcaggctagg gatataacca agccgctccc gaagaatttg 1360
1361 gtatgggaag ctcttcacac tcatggttaa aattaccctt agttcatgta ataatttgag attatgtatc tcctatgttt 1440
1441 gtgtcttgtc ttggttctac ttgttggagt cattgcaact tgtcttttat ggtttattag atgtttttta atatatttta 1520
1521 gaggttttgc tttcatctcc attattgatg aataaggagt tgcatattgt caattgttgt gctcaatatc tgatattttg 1600
1601 gaatgtactt tgtaccactg tgttttcagt tgaagctcat gtgtacttct atagactttg tttaaatggt tatgtcatgt 1680
1681 tattt
                                                                                           1685
```

FIG.5B

```
  1 MAAQIKKYIT SDELKNHDKP GDLWISIQGK AYDVSDWVKD HPGGSFPLKS LAGQEVTDAF VAFHPASTWK NLDKFFTGYY 80
 81 LKDYSVSEVS KDYRKLVFEF SKMGLYDKKG HIMFATLCFI AMLFAMSVYG VLFCEGVLVH LFSGCLMGFL WIQSGWIGHD 160
161 AGHYMVVSDS RLNKFMGIFA ANCLSGISIG WWKWNHNAHH IACNSLEYDP DLQYIPFLVV SSKFFGSLTS HFYEKRLTFD 240
241 SLSRFFVSYQ HWTFYPIMCA ARLNMYVQSL IMLLTKRNVS YRAQELLGCL VFSIWYPLLV SCLPNWGERI MFVIASLSVT 320
321 GMQQVQFSLN HFSSSVYVGK PKGNNWFEKQ TDGTLDISCP PWMDWFHGGL QFQIEHHLFP KMPRCNLRKI SPYVIELCKK 400
401 HNLPYNYASF SKANEMTLRT LRNTALQARD ITKPLPKNLV WEALHTHG                                    448
```

FIG. 6


0.642
Borage Δ6
0.345
(±0.034)
0.642
Synechocystis Δ6
0.128
Arab. chloroplast Δ15
0.027
(±0.011)
0.128
Rice Δ15
0.061
(±0.011)
0.155
Glycine chloroplast Δ15
0.171
(±0.020)
(±0.191)
0.033
Arabidopsis Δ15 (Fad3)
0.184
(±0.005)
0.161
(±0.045)
0.033
Brassica Δ15 (Fad3)
0.085
Borage Δ12 (PI-81)
0.302
(±0.009)
0.085
Arabidopsis Δ12 (Fad 2)
0.438
(±0.043)
0.122
Arab. chloroplast Δ12
0.060
(±0.011)
0.122
0.144
Glycine plastid Δ12
(±0.014)
0.181
Spinach plastidial n-6 (Δ12)
0.223
(±0.027)
0.175
Synechocystis desA (Δ12)
0.150
(±0.014)
0.175
Anabaena Δ12

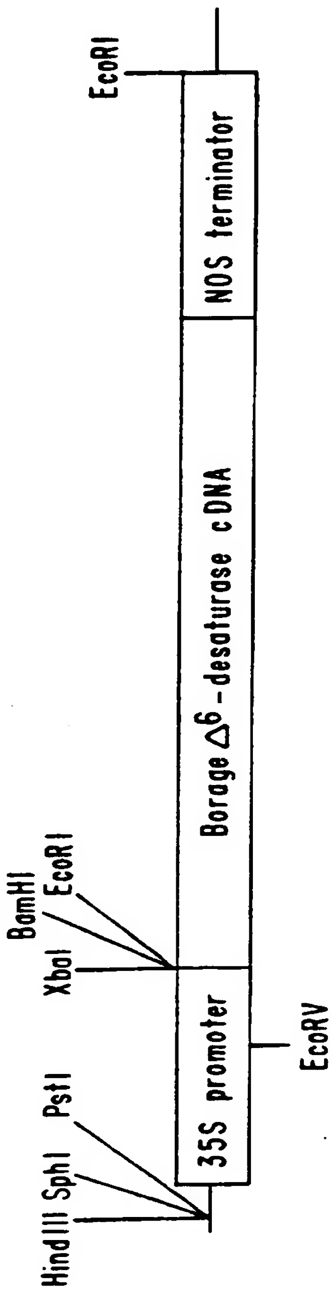

EcoRI
NOS terminator
Borage Δ6-desaturase cDNA
BamHI
EcoRI
XbaI
35S promoter
EcoRV
HindIII
SphI
PstI

FIG. 7

FIG. 8A

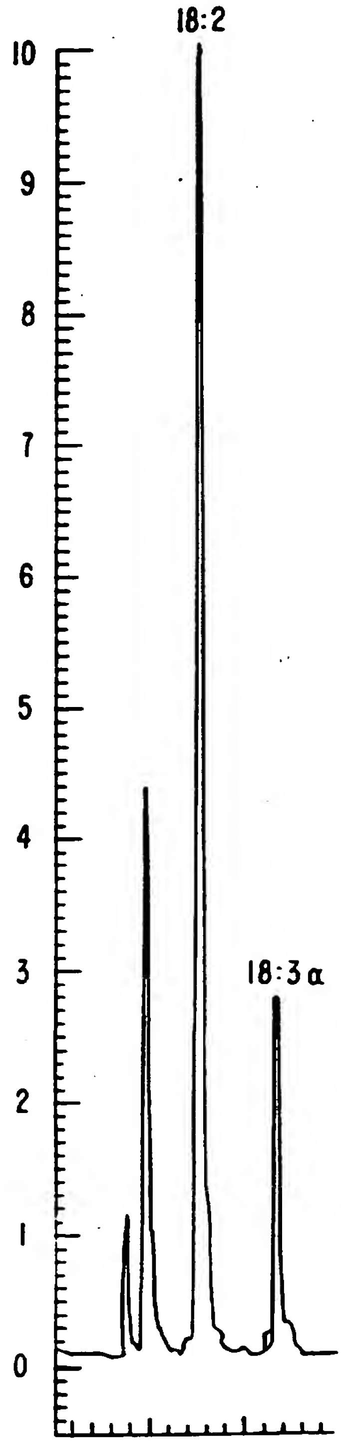


FIG. 8B

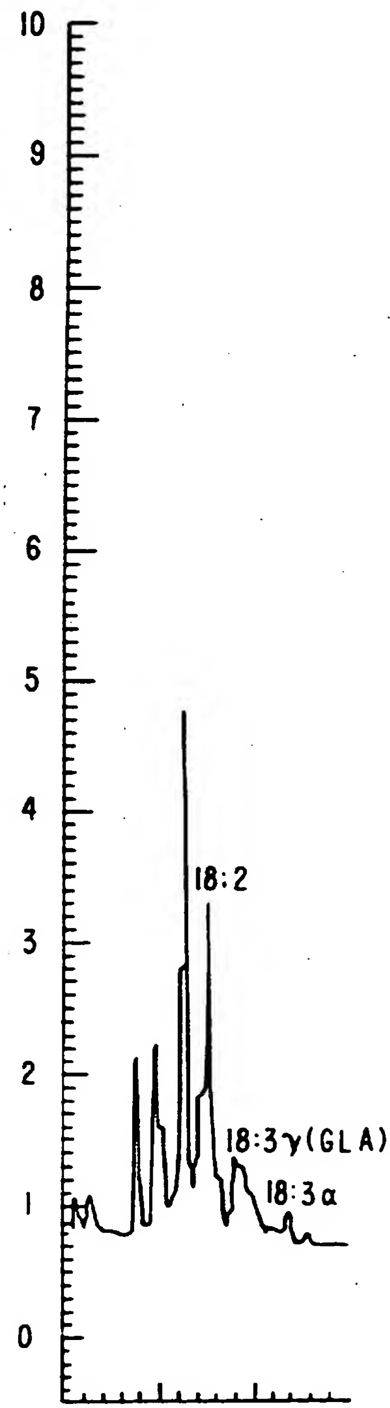


FIG.9A

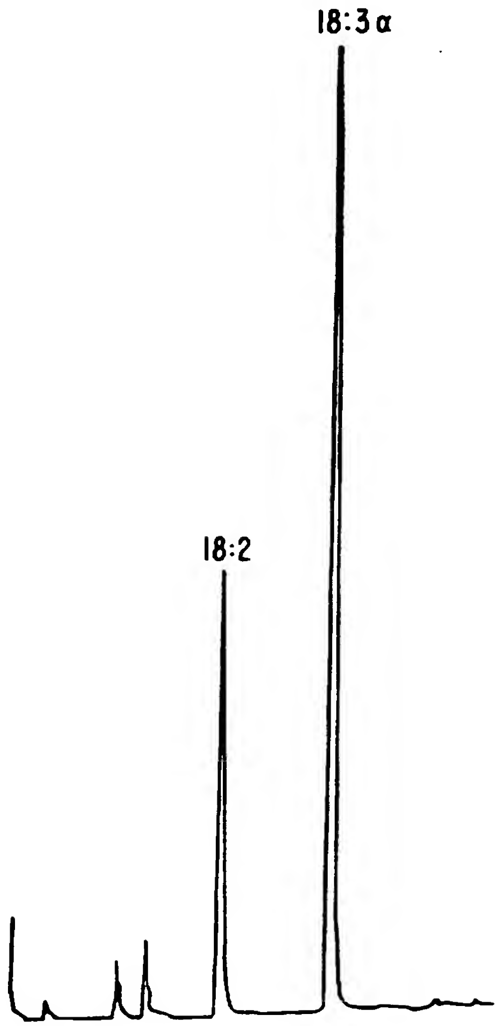


FIG.9B

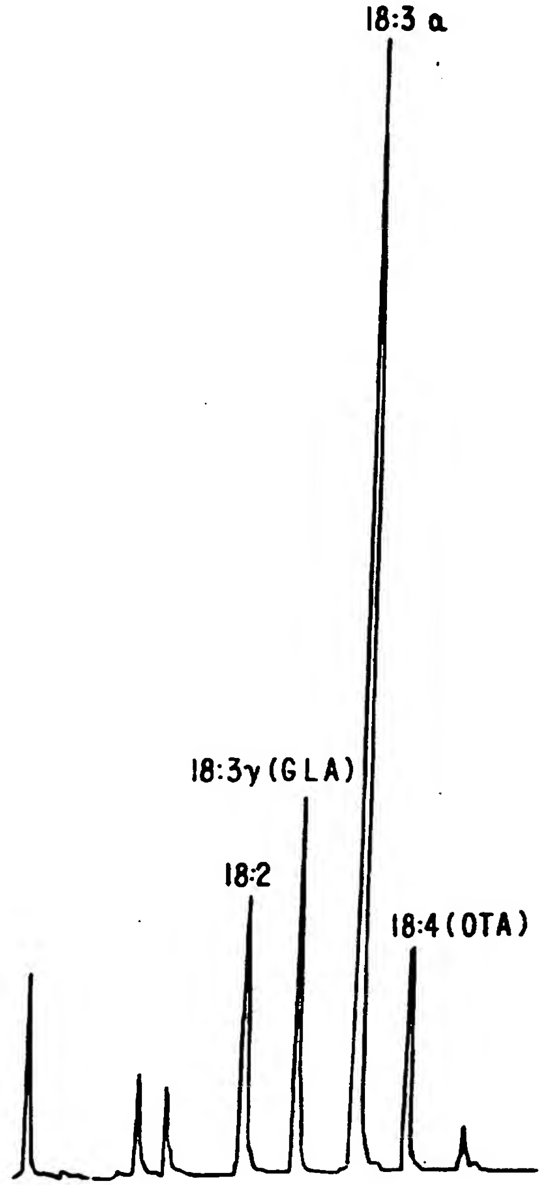


FIG. 10A

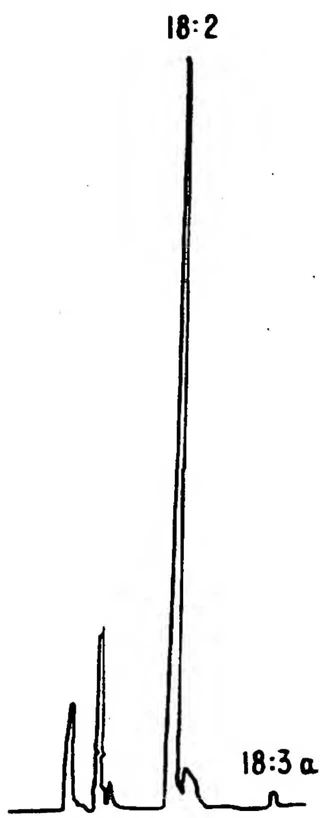


FIG. 10B